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Quality publication is one of the ways to keep science alive, and good journals have a leading role to play in shaping science for humanity! As teachers, we have great responsibilities, we have to advocate our students to accomplish and show them the path to test their mettle in hard times to excel, especially in the post COVID 19 era. Science and its advocates will rise to the occasion and will soon provide succor to the already grief stricken humanity.

We have to fuel our science students with a never say die attitude to let humanity survive!

Amicably yours

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Toll Like Receptors (TLRs) Strategies for the Control of Protozoan and Helminthes Parasitic Infections: An Updated Review

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ABSTRACT

In developing countries, people remain infected with protozoan and helminths parasite contributing to death and economic loss. Toll-like receptors (TLRs) family is considered one of the most major pattern recognition receptors found to be concerned in innate immunity and expressed intracellularly as well as on cell surfaces. They play an essential function in the various protozoans and helminths' ability to activate the host immune system response. These were engaged in producing cytokines and chemokines to promote phagocytosis and the killing of parasites. TLRs have negative regulation strategies to decrease the production of pro-inflammatory cytokines to reduces excessive pathology and reduce tissue damage caused by unregulated TLR activation. The main achievements findings made on the strategies of TLRs negative regulation mediated host response against protozoan and helminthic parasitic infections have been collected using many databases and the research published papers in indexed medical journals. Clarification of the relationship of host-parasites with the TLR pathway would enhance the management method of pathogens immunotherapy. Research in these fields have informed the production of new generation of therapeutics so that science in this field will have a promising future. In this review, we discuss the relationships between most pathogens and the strategies of TLRs to manage infectious diseases in the host immune regulatory network. Furthermore, the promising role that TLRs play in all parasite infections and therapeutic control strategies will also be stressed. Further studies are required to check for interplay strategies that control negative regulation that will provide new guidelines for the therapy of several crosstalk pathogens.

KEY WORDS: FURTHERMORE, PRO-INFLAMMATORY, ESSENTIAL FUNCTION.

INTRODUCTION

The main concern in today's world is of the rising number of cases of protozoan and helminth parasite infections with a high mortality rate (Ropert et al. 2008).

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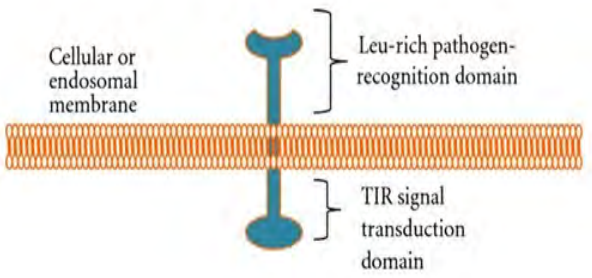
These parasites have the property of immunomodulation, as they have their own strategies to escape from immune responses to survive inside their host for long periods (Maizels et al. 2004). Toll-like receptors (TLRs) are characteristic cellular receptors that can recognize pathogens and their associated molecules through innate infection responses (Kaye and Aebischer 2011). Thirteen TLRs have been currently identified, of which TLR1–TLR9 are retained within human and mice. Nonfunctional TLR10 found in mice whereas TLR11-TLR13 are detected within mice but are lost from human (Takeda and Akira 2015).

In the last decade, the potential role of Toll-like receptors in fighting parasitic infections has attracted much

interest. TLRs serve as the front line of defense response in the host against infecting pathogens by initiating an intracellular signaling cascade to activate an early inflammatory and innate immune response (Sudhagar et al. 2020). This review will cover the main conclusion regarding the mediated reaction between TLRs related protozoan and helminths infections.

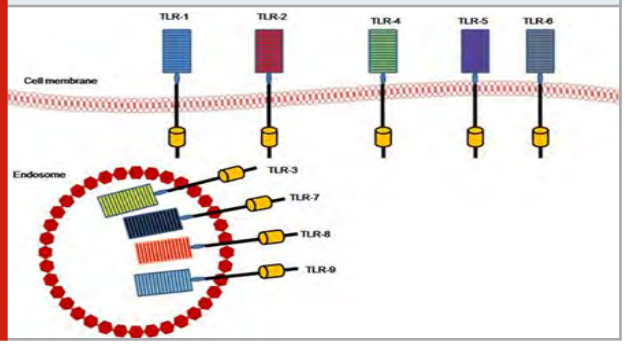
Structure of TLR: All TLRs have two domains: 20–27 extracellular leucine-rich repeats domain that detects pathogen and a Toll-interleukin 1 (TIR-1) domain that interacts with an adapter molecule to set off an immune response (fig. 1). The main differences in TLRs are ligand specificities, signal transduction and subcellular location (El-Zayat et al. 2019).

Figure 1: Schematic diagram of TLR (Muccioli et al. 2012).



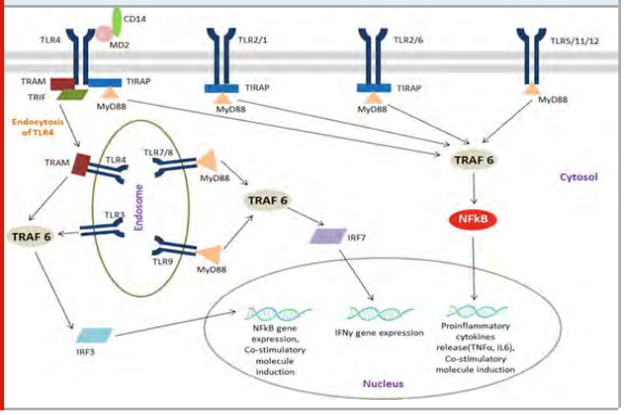
TLRs members: There are 13 mammalian TLR members, 12 in mice, and 10 in humans. The TLR10 mouse gene is not functioning (Ann et al 2016). They are distinguished by repeats of the leucine-rich extracellular domain and an intracellular domain with a retained zone receptor called the Toll/ I L-1 (TIR) domain and the transmembrane domain (Ulevitch 2004). Repeats with Leucine-rich are present in cytoplasmic and transmembrane proteins and participating in the identification, banding, and signal transduction of ligand (Kobe and Deisenhofer 1995). TLRs are functionally subdivided into cell membrane TLRs and Intracellular TLRs or nucleic acids sensors (Fig. 2). All 13 types of TLRs elicit the myeloid differentiation primary response protein 88- (MyD88-) dependent pathways except TLR3 (Ji-Yoon et al. 2020).

Figure 2: TLRS members and their location (El-Zayat et al. 2019).



TLRs role of monitoring parasite infection: Specific pathogen-associated molecular patterns (PAMPs) are identified and interacting with pattern recognition receptors (PRRs) expressed in innate and non-immune cells in foreign organisms. PRRs display the critical function, including immune and non-immune cells, of the innate immunological reaction. They also identify released molecules from damaged host cells called damage-associated molecular patterns (DAMPs). TLR and NOD (nucleotide oligomerization domain-like receptor) recognize intracellular PAMPS and activate signals that induce inflammatory cascades of cytokines to be produced (Mariathasan and Monack 2007 & Takeuchi and Akira 2010).PAMPs interactions with PRRs activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- B).

Figure 3: TLR signaling pathway (Chandrani 2019).



Proinflammatory cytokines such as interleukins (e.g. IL6) and tumor necrosis factor α (TNFα) are released into circulation after infection because of NF-αB migrate to the nucleus (Fig. 3). Such cytokines can regulate the inflammatory reaction through altering vascular endothelial permeability, infiltrate blood cells into inflamed tissues, and develop acute-phase protein production. Certain PRRs monitoring PAMP to recruit IRF3 in addition to IRF7 transcription factors resulting in the development of IFN-β, which play a central role in antiviral response. The innate immune reflex also invokes and induce adaptive immune system activation, which gives the host immune memory towards the invading pathogen (Iwasaki and Medzhitov 2015 & Ji-Yoon et al. 2020).

TLRs are mainly expressed in immune-functioning tissues, including spleen and peripheral blood leucocytes as mast cells, macrophages, and dendritic cells. TLRs found on the plasma membrane excluding TLR3, 7, 8, and 9. Such TLRs are found on the endosome and lysosome membranes with no detectable expression on the surface of the cell (Takeda and Akira 2005). The TLRs, especially surface receptor TLR2 and TLR4, have gained immense importance because they are extremely capable of identifying different molecular patterns from invading pathogens. Such (PRRs) function not only as innate sensors but also as innate and adaptive

immune responses to form and bridge. They also play an important role in regulating the balance between Th1 and Th2 type responses (Mukherjee 2016).

To control adaptive immune responses, the ancient Toll – IL-1 receptor signaling pathway is important. TLR signals through MyD88 pathway leading to activation of MAPK and induces NF-kB to the nucleus which promotes the induction of proinflammatory cytokines (Franco 2017).

Parasites can both activate and negatively regulate TLRs, this mechanism is used by the parasite to suppress the host immune system by interfering with TLRs ' expression and function (Venugopal et al 2009). Moreover, TLR-mediated signal transduction mechanisms and their consequences explain the operation of other immune signaling pathways. TLR signaling deficiency leads to various pathological chronic diseases and manipulation of TLR signaling promises to halt their activity (Yibo et al. 2020).

Table 1. Highlights TLR genes distribution across the genome, the number of amino acids, molecular weight, and their adaptor molecule. Table 1: Toll-like receptors (O'Connell et al 2017).

TLRs	chromosome	Protein a.a	M.W (kda)	Ligand(s)	Sources	Adaptor molecule (Vanhoutte et al 2007)
TLR1	4p14	786	84	Triacylated lipopeptides	Gram-positive and negative bacteria	MAL/MyD88
TLR2	4q31-32	784	84	Bacterial lipoproteins	Gram-positive and negative bacteria	MAL/MyD88
TLR3	4q35	904	97	Heat shock protein 70 dsRNA	Mycoplasma and Host/ self Viruses and Host/self	TRIF
TLR4	9q32-33	839	90	*Lipopolysaccharide*	Gram-negative and bacteria	MAL/MyD88 and
				Heat shock proteins	Host/self	TRAM/TRIF
TLR5	1q33-42	858	91	Flagellin	Flagellated bacteria	MyD88
TLR6	4p14	796	91	Diacylated lipopeptide	Mycoplasma	MAL/MyD88
TLR7	Xp22	1049	121	ssRNA	Virus	MyD88
TLR8	Xp22	1041	120	ssRNA	Virus	MyD88
TLR9	3p21		116	Unmethylated CpG DNA	Bacteria and Self	MyD88
TLR10	4p14	1032	95	Unknown	Unknown	Unknown

TLRs distribution in different immune cells: TLRs are receptors expressed by different types of immune cells, a major component of innate immunity. TLR 1,2,4,5,6,7,8 is shown in neutrophils and monocytes/macrophages, and the dendritic myeloid cell expresses TLR 2.3,4,7,8. As well as, B lymphocytes generate TLR 1,3,6,7,9,10 while T lymphocytes (Th1/Th2) express TLR 2, 3, 5, 9. Three types of TLR can express by T lymphocytes (regulatory) are TLR2,5,8. Expression of TLR2,4,5,7,8,9 usually on mononuclear peripheral blood cells (Chandrani et al 2019).

TLRs and parasite infection: TLRs have potential as therapeutic targets, either alone or in combination with standard immunotherapy and pharmacotherapy. In recent years, TLR antagonists or agonists from their negative regulators have been investigated as vaccine adjuvants to improve tumor, allergy, and infectious disease immune response. TLRs regulate downstream pathways involved in adaptive immune responses by influencing multiple antigen-presenting cell (APC) functions (Kanzler et al 2007). TLRs may be used to differentiate parasites from the self by macrophages and dendritic cells. Furthermore, TLR families are known to be combine with the pathogen and activating downstream signal transduction pathways. Moreover, TLRs have been demonstrated to be important for protozoan parasite resistance and identification of their components (Ghartey et al. 2020).

Infection by *L. major* in TLR4 mice leads to low parasite replication that correlates with higher levels of inducible nitric oxide synthase. Hence, TLR4 competence could resolve cutaneous lesions and control parasite growth (Kropf et al 2004). TLR2 forms heterodimers with TLR1 or TLR6 and modulates downstream signaling pathways. LPG's *L. major* is involved in modulating the expression and function of TLR9. Mice infected with L. major increases the expression of TLR1 and TLR2 but TLR6. The association TLR2-TLR2 increases but the association TLR2-TLR6 decreases. The difference between TLR2 and TLR1 or TLR2 and TLR6 in the development of heterodimers results in a functional duality of TLR2. Pegylated bisacycloxypropylcysteine, a diacylated lipopeptide ligand of TLR6, plays a host-protective role against experimental *Leishmania* major infection (Pandey et al 2014).

Mice deficient in either TLR2, 4, or 9 are more susceptible to *L. major* infection than TLR9-deficient animals. The TLR9 deficiency inhibits the response of curative Th1 (Abou-Fakher et al 2009). TLR9 seems to play an important role in infection by *L. major* or *L. infantum*

(Sacramento et al 2015). On the other hand, TLR11 and TLR12 play an important role in a L. major infection by reducing the parasite burden, increasing the IFN-γ level, and decreasing IL-4 production (Shukla et al 2018). TLRs 2, 3, 4, and 9 play an important leishmanicidal role; however, TLRs can facilitate replication of *Leishmania* and make the recipient more susceptible to infection in the late stages of infection (Dalia 2015). Recent research has shown that TLR4-IRF1 inevitably results in production of IFN-β as a tool to damp the chronic inflammatory process (Sacramento et al. 2020).

Table 2. Functional immunobiology of different TLRs against array of pathogens.

Parasite Protozoa	Targeted receptor	Effector Function
Leishmania major	-TLR2	-IFN-γ, TNF-α, and NFκB (Hawn et al 2002)
	-TLR2, 4	- IL-12, TNF- α, IFN-γ and NO
	- TLR9	(Becker et al 2003 & Abu-Dayyeh et al 2010) - IFN-γ (Abou-Fakher et al 2009)
Leishmania braziliensis	-TLR4	-TNF-α and IL-10 (Galdino et al 2016)
Leishmania infantum	-TLR9	-IFN-α/β and IL-12 (Schleicher et al 2007)
	-TLR4	-IFN-β and IRF1 (Sacramento et al 2020)
Leishmania mexicana	-TLR2, 4	-TNF-α, IL-1β, IL-12 p40, IL-12 p70, and IL-10 (Rojas et al 2014)
	-TLR9	-TNF-α and IL-12 (Martínez et al 2008)
	-TLR2	-Block IFN mediated (NO) production (Faria et al 2014)
Leishmania donovani	-TLR2	-TNF-α, IL-12, and IFN-γ, NO (Srivastava et al 2013)
Leishmania panamensis	-TLR1,2,3, 4	-TNF-α (Gallego et al 2011)
Toxoplasma gondii	-TLR11	- IL-2 (Yarovinsky et al 2005)
	- TLR11	- IL-12, IFN-γ (Yarovinsky 2014)
	- TLR4	- TNF-α Reduce IL-10 (Gowda 2007)
Trypanosoma cruzi	-TLR2, 6	-NF-κB and IL-8, TNF-α, NO (Bott et al 2018)
	-TLR2	- (IL-12), (NO) and (TNF) (Aoki et al 2012)
	- TLR4	- IL-12, TNF-α NO (Coelho et al 2002)
Plasmodium falciparum	-TLR2, 4	-TNF-α NF-κB, and IFN-γ (Hisaeda et al 2008)
	-TLR9	-FN-γ (Matthew et al 2007)
Entamoeba histolytica	-TLR9	-TNF-α (Ivory et al 2008)
	-TLR2 ,4	-NF-κB ,(IL)-10, IL-12, (TNF)-α, and IL-8 (Maldonado et al 2005)
	-TLR2, 4	-TNF IL-12,IL-10,and NO (Wong et al 2010)
Giardia lamblia	-TLR2	-IL-12 p40, TNF-α, and IL-6, IFN-γ (Li et al 2017)
Helminths		
Ascaris lumbricoides	-TLR2, 4	-IL-10 and TGF-β (Kane et al 2004) - IL-10 (Magdalena et al 2019)
Brugia malayi	-TLR2,4, 9	-TNF-α, IL-6, and IL-10 (Faria et al 2014)
Toxocara canis	-TLR2	-IL-4, IL-5, and IL-13 (Ludwig et al 2012)
Acanthocheilonema viteae	-TLR4	-Block of IL-12 and TNF-a Production (Diaz and Allen 2007)
Taenia crassiceps	-TLR2, 4	-IL-6 (Correale and Farez 2009)
Schistosoma mansoni	-TLR2	-IL-1β, IL-6, IL-12, and TNF-α (Du et al 2014)
	-TLR2, 4	-IL-10, TGF-β (Kane et al 2004)
Fasciola hepatica	-TLR3	-IL-10 and IL-4 (Carranza et al 2012)
Trichuris trichiura	-TLR4	-TNF-α (Donnelly et al 2010)
Trichinella spiralis	-TLR4, 9	-IL-10 and TGF-β (Sin et al 2015)
Trichomonas vaginalis	-TLR4	-NF-κB (Im et al 2016)
Naegleria fowleri	-TLR4	-IL-8, TNF-α, IL-1β (Martínez et al 2018)

Two TLR1 polymorphisms have been evaluated, rs4833095 (Asn248Ser) and rs5743618 (Ser602Ile) for their association with *P. falciparum* infection and placental malaria susceptibility (Hamann et al 2010). TLR4-Asp299Gly and TLR4-Thr399Ile variants have been found to give a higher risk of severe malaria. TLR4 polymorphisms, infectious diseases, and evolutionary pressure during the migration of modern humans (Ferwerda et al 2007). Only certain adaptive immune responses need to be triggered by TLRs (Barton and

Medzhitov 2002).Potential associations between TLR2 and TLR4 mRNA expression and cytokine and nitric oxide (NO) production have been studied with *L. chagasi.*

While in the early stage of infection, the mRNA expression of TLR2, TLR4, IL-17, TNF-α, and TGF-β increases, it decreases in the late stage, correlating with parasite load. At the peak of infection, the mRNA expression of IFN-π and IL-12 declines (Cezario et al 2011). Mice were infected with L. braziliensis showed low levels of cytokine IL-12p40, leading to greater infection by L. braziliensis and a limited expansion of CD4+ T cells that produce IFN-γ and IL-17 during infection. In contrast, TLR2 mice were more immune to infection due to increased IFN-α development. Consequently, TLR2 seems to have a regulatory role in modulating the immune response to *L. braziliensi* (Vieira et al 2013).

TLR6 S249P's single-nucleotide polymorphism can be a risk factor for malaria development. (Fabiana et al 2008) TLR5 the single-nucleotide polymorphism S180 *L protects* against malaria, while the single-nucleotide polymorphism of R392 stop codon increases susceptibility to the same disease (Khor et al 2007).The susceptibility can be modulated by a TLR4 polymorphism rs4986790 toward a *P. vivax* infection (Rani et al 2018). TLR4 A299G, TLR6 S249, and TLR 9-1486C/T influence the levels of circulating cytokines IL-6, IFN-γ, IL-12, IL-10, and IL-4 during a P. vivax infection. (Costa et al 2018) TLR9 1237C/C correlates with acute parasitemia during a P. vivax infection (Costa et al 2017). A protein resident in the endoplasmic reticulum, UNC93B1, is essential to hosting resistance to *T. cruzi* and *T. gondii* (Melo et al 2010). TLR2 blocked Giardia infection and reduced the burden of parasites compared to the control that infected with the same protozoan (Li et al 2017).

When TLR receptors bind to the ligands on the surface of dendritic cells of the *S. mansoni, A. lumbricoides,* and *T. trichiura* parasites, expression of co-stimulatory molecules (CD40, CD80, and CD86) and pro-inflammatory mediator synthesis Increase, such as IL-12 and TNF-α, evokes the response of Th1 lymphocytes (McSorley and Maizels 2012). TLR2 is necessary for the priming and expansion of active Regulatory T cells during schistosomiasis (Barton and Medzhitov 2002).In the early stages of infection, mRNA expression of TLR1, TLR2, TLR3, TLR4, and TLR9 is regulated for intestinal parasites.

During the adult stage of infection with *T. spiralis*, TLR1 and TLR4 activate the signaling pathway dependent on MyD88. The expression of TLR2/4 during infection could be closely associated with immune responses mediated by Treg cells and greater expression of cytokines such as IL-10 and TGF-β. (Kane et al 2004) During an *S. Mansoni* infection, TLR2 and TLR4 restrict immune response activation while that of TLRs 1, 3, 7, and 8 is decreased after deposition of the egg (Dalia 2015). The deficiency of these TLRs, therefore, facilitates the removal of adult worms (Pasare and Medzhitov 2003). Table 2; contains a list of the major pathogens involving TLRs and cytokines targeting these parasites.

TLRs Negative regulation: TLRs down-regulation is a strategy that protozoa use to avoid immune responses. It has been shown that protozoan parasites such as Trypanosome spp and Entamoeba histolytica inhibit the immune response by reducing the expression of TLR2. Similarly, B. malayi significantly reduced the mRNA spreading of TLR3,4,5 and 7 from DCs that derived from monocyte (Maldonado et al 2000). The expression of TLR1,2,4 and 9 in B cells has decreased in filarial infected individuals. (Babu et al 2005). During TLR signaling, T cells play an important function as T cells release many of the TLRs. Upon induction by B cells and monocytes, T cells express lower levels of TLR1, 3, and 4 during lymphatically infected patients (Babu et al 2006). *L. donovani* has developed strategies for survival that suppress the pro-inflammatory reaction created by TLRs by suppression of TLR2, 4, and activation of IL-12p40 and IL-10 production (Chandra and Naik 2008). *G. lamblia* helps to reduce the development of TLR3 signaling pro-inflammatory cytokine. *S. mansoni* and *F. hepatica* have a restrained effect on the ripeness of DCs induced by TLR ligands to generate IL-12 less than those that are only activated with TLR4 ligand (Rodríguez et al 2015).

S. mansoni stimulates IL-12 development or increases CD80, CD86 in addition to MHC class II surface expression in DCs. It decreases the development of IL-12, IL-6, and TNF-α, as well as the release of CD80/86 molecules, which can repress the Th1 response and enhances the Th2 response evolved by LPS (79). TLR2 and TLR3 are needed to shape the immune response during murine schistosomiasis and to increase the Th1/Th2 immune response balance (Vanhoutte et al 2007).Low T. cruzi virulence strain induces relatively high TLR4 expression and elevated pro-inflammatory cytokine rates such as IL-12 and TNF-α. On the other hand, it maintains low TLR4 expression and decreases TNF-α development (da Costa et al 2014). In addition, the expression of TLR2 and TLR4 in the brain of the mice was increased after Acanthamoeba sp infection in contrast to animal control (Wojtkowiak et al 2016). Acanthamoeba sp, on the other hand, generates high mRNA expression and high levels of TLR2 in animal brains at 2, 4, 8, 16, and 30 days after infection compared to uninfected mice (Wojtkowiak et al 2018).

Negative TLR regulation may reduce pro-inflammatory cytokine production to control the balance of Th1/2 cells and to modulate the expression of TLR4 that can protect the host from autoimmune development (Terrazas et al 2013). Most helminth pathogens operate the downregulating TLRs to improve the immune system and interfere with multiple gene expression linked to the transduction pathway. Several studies show that continued response to helminth antigens leads to control the response of cells to PAMPs from these parasites and suggesting a poor immune response in helminth

infected individuals (Kane et al 2004).Moreover, latest results suggest that TLR4 obstructs the IRF1 and IFN-β immune response to avoid immunopathological disease (Sacramento et al. 2020).

Current treatment design considerations and Conclusion: Recent studies have shown that TLRs are likely to succeed in combating various parasites by activating signals pathways and their inhibition strategies offer great promise as an entirely new class of biologics for the treatment of inflammatory diseases (Shepherd et al 2015 and Chandrani et al 2019). Moreover, TLR ligands essential for vaccine adjuvant activity which should anticipate adjuvants that may improve the effectiveness of vaccines or have the effects of antiparasitic. The strong interest of biological researchers in TLRs will surely lead to desperately needed parasite therapies.

TLRs have many types of actions that can play a major role in the pathogen immune reaction. Every TLR plays a crucial role in this response. The inborn immune sensors encoded by germline which identify preserved parasite structures and induce signaling cascades that increase the development of inflammatory mediators by macrophages, neutrophils, dendritic cells, and other types of cells. TLRs are also able to trigger inflammatory cytokine production, which in many diseases plays a pathogenic role. Such processes activate mechanisms of innate host defense immediately, as well as trigger and organize the adaptive immune response.

Studies on infectious diseases identified by TLRs helped to understand the fundamental mechanisms of innate immune responses to most parasites. Parasites interfere with the immune response in order to promote their host survival. TLRs polymorphisms play an important role in rising host parasitemia and ligands of TLRs come from PAMPS and host DAMP.Ligand-mediated toll-like receptor activation (TLRs) not only causes inflammation but also suppress immunity, which is a new area of research. Uncontrolled activation of TLR can lead to severe inflammatory conditions and tissue damage. Through developing strategies for stabilizing negative regulation molecules it is possible to reduce the incidence of infectious diseases. Additional signaling channels of the innate immune system affect the negative regulation of TLR signaling. Consequently, TLRs negative regulation results in a lower output of pro-inflammatory cytokine and limits excessive pathology. Additional studies are necessary to describe the control in this system through these interactions. This upcoming research will offer new guidelines for the therapeutic treatment of several pathogenic infections.

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Thyroid Lymphoma Treated with Combined Modalities: A Case Report With Literature Review

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ABSTRACT

Primary thyroid lymphoma (PTL) is a rare disease, accounting for 5% of all thyroid malignancies. Diffuse B-cell lymphoma (DBCL) is the most common type of PTL, about 50–80% of PTL is DBCL. The case of an 81year-old female, known case of diabetes and heart disease, is presented with large neck mass on the left side, pushing the airway and not associated with B symptoms. On examination, 9 cm neck mass detected on the left side and no cervical lymph nodes. CT scan showed left sizeable soft tissue mass measures 8.7 cm, compressing the trachea. Fine-needle aspiration (FNA) was inconclusive, and the biopsy revealed DBCL, which indicated PTL. PTL originated from B cells, especially DBCL, there is no consensual therapeutic scheme for PTL; each case must be evaluated and treated separately. Treatment included surgery or Radiotherapy to achieve reasonable local disease control, combined with chemotherapy for control spread disease, and improving the outcome. The five-year Over-all survival (OS) for PTL in the literature range from 35% to 100%. PTL considered to be tough to diagnose in the preoperative period; Each case diagnosed with PTL should be evaluated and treated separately, depending on the staging after the diagnosis. Well-organized care and, combined modalities revealed effective management.

KEY WORDS: PRIMARY THYROID LYMPHOMA, DIFFUSE B-CELL LYMPHOMA, RADIOTHERAPY, CHEMOTHERAPY.

INTRODUCTION

Primary thyroid lymphoma (PTL) is an unusual disease; it is mainly occurring in middle age and female predominant (Walsh 2013, Graff-Baker2010). Thyroid malignancies have 5 % PTL; whereas, Extranodal lymphomas have 7 % PTL. (Widder 2004, Stein 2013) B-cell lymphoma (DBCL) is common in PTL; it accounts for 60–80%. (Gupta 2005– Thieblemont 2002, Onal 2011). It is very curable disease, therefore early detection and correct treatment is essential (Gonçalves 2018). In the past, before the introduction of FNA biopsy, surgical resection was the mainstay treatment for thyroid lymphoma (Graff-Baker 2010, Meyer-Rochow 2008). Later the role of surgery has reduced as resection of thyroid is no longer required for diagnosis, histopathological evaluation supplemented by Immunohistochemistry (IHC) is the gold standard for the diagnosis of PTL (Kakkar 2019). It is challenging to diagnose primary thyroid lymphomas because of their rare occurrence (Acar 2109), and its management requires the involvement of an interprofessional team of specialists from different departments (Kesireddy 2020).

Several studies reported improved outcomes in PTL with the combined modality of both radiation and chemotherapy (Vardell2019, Onal 2011, Watanabe 2011). It still remains unclear what optimal treatment methods should be adopted with PTL patients.In this study, we report a case of primary thyroid lymphoma confirmed on histology, presented at the multidisciplinary tumour board at King Abdul-Aziz university hospital in Saudi

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Arabia and treated by bimodalities chemo-radiotherapy as well we reviewed the relevant literature in order to summarize the management scheme of PTL.

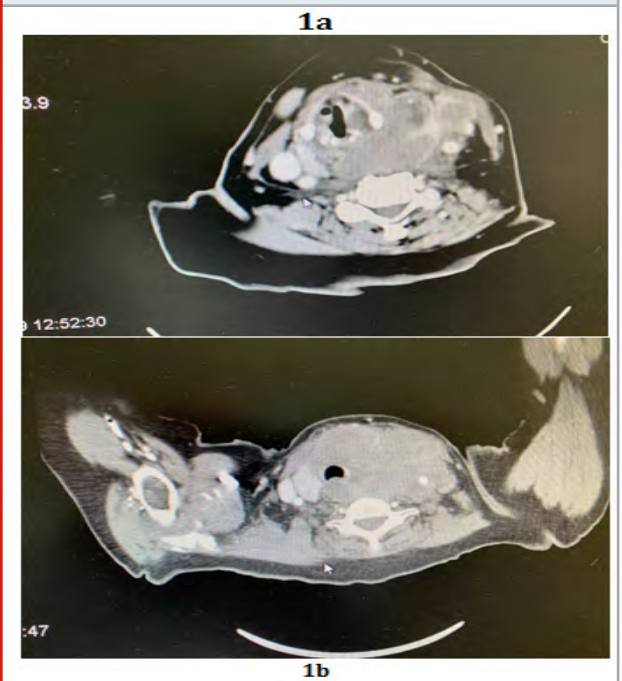
Case Presentation: Eighty-one years old female known case of Diabetes (DM)and, heart disease on medication, there was no family history of thyroid cancer neither previous neck irradiation. She presented with a considerable neck mass on the left side; it was rapidly progressing in size for the last six months, pushing the airway. She complains of dysphagia mainly to solid food; subsequent fine needle aspiration cytology (FNAC) performed from the thyroid gland; and, it was negative for malignancy, followed by Excisional biopsy on 24-2-2018, which showed high-grade B cell lymphoma, consist with DLBCL.Bone marrow biopsy was done on 4-4-2018 and revealed no malignancy.

Diagnostic assessment: Neck ultrasound on 14-2-2018

It showed; enlarged left thyroid lobe; measuring 9.3 x4.6 x 5cm and replaced by a sizeable heterogenous mass with no calcification. The right thyroid lobe appears homogenous, with normal vascularity, no nodules, or masses.

CT Scan of the Neck on 22-2-2018: It revealed; a large infiltrative left thyroid lobe mass with an area of necrosis. The mass is extending superiorly; to the level of the hyoid bone. It is reaching the thoracic inlet inferiorly by 1.3 cm below the sternal notch. The tumour measured 8x8.7 x6.4 cm.; and compressing the trachea, with narrowest diameter measures 0.9 cm. Figure 1a and b. No cervical lymph nodes determined in the CT scan.

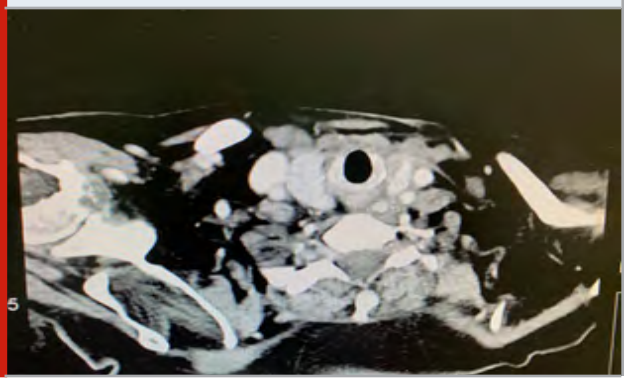
Figure 1a,1b showed an axial neck computed tomography images demonstrate a large ill-defined mass infiltrating the thyroid gland.



Biopsy on 24-2-2018: Showed Diffuse large B-cell lymphoma, the tumour cell CD20 positive and, CD10, CD3 negative. Staging works up (CT chest and abdomen) were negative for metastasis. Although (PET) scan is essential at diagnosis, particularly in patients with (DLBCL), unfortunately, we don't have a PET scan at our centre.

Follow-up and Outcome: The patient received six cycles of chemotherapy (CVP and rituximab), the last chemo was on 23-10-2018 followed by radiotherapy (RT) treatment, a total dose of 36 Gy/18 fractions, 2Gy per fraction, delivered using three dimensional conformal Radiotherapy. The clinical target volume (CTV) was encompassing the whole thyroid. The patient completed radiotherapy treatment on 26-12-2018 with no significant toxicity or treatment interruption. On follow up by clinical exam, and computed tomography (CT) scan; we noticed a decrease in size of the neck swelling. CT neck after chemotherapy showed interval disease regression. The mass measured 3.5 cm x 2.5cm. Ultrasound neck on follow up,25-09-2019 showed left thyroid lob measures 1.5 x0.5x1.3 cm. No recurrence or metastasis detected in our case during the follow-up period; we assessed the patient both clinically and radiologically. figure (2)

Figure 2: CT Neck on follow up, showed no recurrence.



DISCUSSION

Typically, the thyroid gland does not include lymphoid tissue and most PTL are non-Hodgkin's lymphomas, whereas Hodgkin lymphoma and T-cell NHL are rare, (Chen 2014). PTL is accountable for 2.5% of all lymphomas, (Alzouebi 2012), (Campo 2006). PTL has four types; include diffuse large B-cell lymphoma (DLBCL), marginal zone lymphoma (MALT), follicular lymphoma (FL), and Extranodal small lymphocytic lymphoma, (Derringer 2000).Diffuse B-cell lymphoma (DLBCL) is the most common type of lymphoma in general, (Katna 2013). DLBCL is usually composed of sheets of atypical lymphoid cells has an irregular nuclear membrane, and granular chromatin, with round nucleoli and scant cytoplasm, (Cozzolino 2016). Several studies have documented that Hashimoto's thyroiditis (HT) histologically seen in 90% of cases of PTL (Widder 2004), is due to the presence of lymphocytes in HT, and the chronic antigenic stimulation, which predisposes

malignant transformation of these lymphocytes, (Green 2006, Kossev 1999). It is very challenging to distinguish Hashimoto's thyroiditis from PTL on fine-needle aspiration (FNA), (Pasieka 1998 and Chai 2015).

PTL presented as a painless thyroid mass that enlarged and caused symptoms associated with the compression, such as dysphagia, hoarseness of the voice and, dyspnea, (Wirtzfeld 2001). Diffuse neck swelling recognized during the physical examination; neck ultrasound and CT scan. (Katna 2013) Ultrasound is considered an initial diagnostic modality for PTL; By ultrasound findings, PTL can be classified as a nodular, diffuse, or mixed subtype (Ota 2006); however, it is difficult to distinguish PTL from other thyroid diseases. Wang identified in his study that positron emission tomography-computed tomography [PET scan /CT] is a practical image which could distinguish between PTL and chronic thyroiditis, the SUV max was substantially higher; and, the CT density was lower in PTL in comparison with chronic thyroiditis, (Nakadate 2013 Wang 2014).

Ultrasonography plays an important role in monitoring and follow-ups after chemotherapy in patients with PTL, (Li 2019). The gold standard for diagnosing PTL depends on biopsy, which has higher diagnostic accuracy, and the management depended on histology, patient performance status, and co-morbidities (Sharma 2016). Fine-needle aspiration (FNA) has an essential role in diagnosing PTL, with an accuracy of 25%–90% (Gupta 2005, Nguyen 2005). Still, it has a limited effect to differentiate between thyroid lymphoma, and thyroiditis, which results in increased false-negative outcomes from sampling error. Sangelli et al documented 4 out of 10 cases MALT thyroid lymphomas discovered by using FNA biopsy in comparison to six out of 7 cases of thyroid DLBCL. (Sangalli et al 2001). Core biopsy is more sensitive than FNA, and still required for confirming the definitive diagnosis of PTL(Vigliar 2013, Sarinah 2010).

Further testing is needed such as flow cytometry, which examines CD markers and improves sensitivity and specificity; (Adhikari 2015). DLBCL has negative CD19-, CD20-and positive CD45-; while MALT lymphoma has CD5-, CD10-, and CD23-negative; and CD19+, CD20+, (Higgins 2008). Recently a study by Travaglino et al identified clinical features associated with high pathological grade in primary thyroid lymphoma includes Age≤55, female sex, lymph node involvement, compressive symptoms and absence of lymphocytic thyroiditis (Travaglino et al., 2020). Once the PTL diagnosis is confirmed by otolaryngologist and the pathologist, the radiation, medical oncologist, and an endocrinologist should participate in the case; further workup and treatment should begin immediately, (Kesireddy 2020).

Treatment option includes surgery, radiotherapy, chemotherapy, and combination treatment with chemotherapy and radiotherapy, (Foppiani 2009). No significant prospective studies are showing the therapeutic approach of PTL; most physicians intend to do multidisciplinary loom. The management of patients with PTL varies according to histology and the stage of the disease; therefore, the concern, in this case, was to differentiate PTL from secondary thyroid lymphoma (STL). The primary thyroid lymphoma common in females, occurs in older women between 6-7th decades, (Pedersen1996). Patient presented with rapidly enlarged painless mass that associated with cough, hoarseness, dysphagia, dyspnea, and pressure symptoms, (Derringer2000). Where as the secondary thyroid lymphoma it occurs in the middle-aged population around 42 years, mostly disseminated disease metastasized to the thyroid and has a higher mortality rate in contrasting with primary thyroid lymphoma which occurs in early stages disease, (Takashima 2000).

The majority of cases diagnosed with stage IE (30–66%) or stage IIE (25%–66%).(Graff-Baker 2010, Stein2013,, Onal 2011, Campo2006, Watanabe2011). Only 20 % present with advanced stage IIIE and IVE disease.The staging of PTL performed according to Ann-Arbor classification. (Table 1)(Carbone 1971). Our patient staged as 1E with no B symptoms and Lymphoma International Prognostic Index (IPI) Score=1, (Amy2020).

Table 1. Ann-Arbor classification

Stage 1E Confined to the thyroid gland
Stage 2E Locoregional lymph node involvement in addition to
thyroid gland
Stage 3E Involvement of lymph nodes located on both sides of
the diaphragm
Stage 4E Disseminated disease
A: Absence of symptoms
B: At least 10% body weight loss over six months, fever without an infection over 38 degrees, recurrent night sweating.

PTL is highly sensitive to radiation; it is responding rapidly to combined modalities with induction chemotherapy followed by involved-field radiotherapy. (Alzouebi2012, Yahalom2015).RT dose, treatment field, and technique vary, the doses range from 36Gy to 45Gy depends on volume and residual post-chemotherapy and the intent of treatment. (Yahalom2015, Kakkar 2019). They used involved field by including the whole thyroid with or without regional lymph nodes, either with 3D conformal, or intensity-modulated radiotherapy (IMRT). In our case, 36Gy used as the patient was elderly.The chemotherapy that used are usually the same chemo as in Non-Hodgkin Lymphoma, CVP (cyclophosphamide, vincristine, prednisolone), and;CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone), and R-CHOP. (Miller1998). Rituximab is immunotherapy (monoclonal antibody) agent which often added to chemotherapy, acts against CD20, and showing a promising result in managements of DLBCL in the form of improved disease-free and overall survival, (Nakadate 2013, Coiffier 2002, Pavlidis 2019).

Our patient received six cycles of chemotherapy (CVP and rituximab); since she had a cardiac disease, and CHOP is known to induce cardiotoxicity in elderly patients who appeared to be at higher risk, (Limat 2003). Several studies have reported that PTL has an excellent prognosis when treated with combined modality, 5-year OS, and DFS in Ha et al. was 64% and 76%, (Ha et al 2001). Further literature by Matsuzuka et al.; reported eight-year over-all survival (OS) 100%for patients who treated with chemo and, radiation, (Matsuzuka et al 1993). Doria et al.; showed that combined therapy had significant improvement in local control over a single modality treatment (surgery, radiotherapy, and chemotherapy) and lowered the relapse rate, (Doria et al., 1994).

The majority of the literature on PTL presented as case series and case reports, with paucity of retrospective studies for this disease. However recently the National Cancer Database (NCDB) reported a large retrospective study on PTL; the results showed patients who were treated with multiagent chemotherapy, have the best survival of all treatment modalities. Surgical resection and radiation therapy both show a significant survival benefit, (Vardell, 2019).The prognosis of PTL determined by disease subtype and histology. (Sakorafas 2010) There are several factors predict a worse prognosis in PTL, includes, age greater than 80 years, advanced stage, size of the tumor more than 10 cm, rapid tumor growth, (mediastinal do worse), and elevated LDH, (Tupchong1986, Aozasa 1986 Onal 2011, Kesireddy 2020).

We calculated the International Prognostic Index (IPI) score for this patient and, it was 1, 4-year progression-free survival is 80 %.(Sehn 2007).The life expectancy for DLBCL in 5 years is 75% and in MALT lymphoma is 96 %. The recurrence classically appears within the first four years. Life expectancy by stage in five years is 86% for stage 1E, stage 2E is 81 %, and 64% for stage 3E-4E (Graff-Baker 2010). A study by Vardel et al. (2019) revealed that the median survival of PTL is 11.6 years and, five years overall survival (OS) is 75%. Although the rarity of PTL; it considered to be a curable condition with any histological subtype. Rituximab provided an excellent outcome in most cases; our patient achieved a complete response with no complication, (Coiffier 2002). Our case also showed that tissue biopsy was the tool for the confirmation of PTL, which revealed DBCL in-addition to the clinical presentation and, the-radiological features of PTL, while FNA was negative for malignancy. PTL is easily misdiagnosed therefore, it's very necessary to analyze the diagnosis methods and treatment strategy of PTL to clarify the guide of diagnosis and treatment, (Wang 2020).

CONCLUSION

Primary thyroid lymphoma is an uncommon disease and considered to be tough to diagnose in the preoperative period; however, it is curable with suitably overall survival. Each case diagnosed with PTL should be evaluated and treated separately, depending on the staging after the diagnosis, which influences the treatment. Our case report revealed effective management with combined multiagent chemotherapy and radiotherapy treatment. PTL has a good prognosis if the patient received well-organized care from all the specialists promptly.In the future, extensive studies are required to standardize the therapeutic approach; and establish ideal PTL treatment guidelines, furthermore we should consider comparing treatment modalities of chemotherapy alone vs chemoradiation.

Ethical approval: This is a case report, without clinical research involved.

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A Mini Review on Immune System and Immunological Diseases: Properties, Classification and Evolutional Aspects

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ABSTRACT

The fundamental definition of immunity is the organism's ability to resist the invasion of micro-organisms and dangerous substances and even unaltered host components. The immune system defends our bodies against the danger of the threat by communicating with chemicals and cells, which are incredibly complex to separate, kill and eliminate potential threats and also can cause disease under some circumstances. The immune system made up of certain and non-certain systems. The non-certain immune system is consisting of two different types of mechanisms (cellular and non-cellular). A certain immune system is completely assisted by lymphocytes and agranular leukocytes. Immune dysfunction or reduced immune competence may be caused by a number of factors such as fatigue, malnutrition and simultaneous infections. Immune-related disorders include allergy, asthma, autoimmune conditions, auto-inflammatory syndromes, and immune deficiency syndromes. Different functions of different immune systems seem to have been the primary driving force in the development of immune systems as a pathogenic diversity defense mechanism. Evolution of immunity exists over various occasions of the host population lifespan and related to the evolution of pathogen and the frequency shifts in a person's lymphocyte clone during an infection. The data bases, books and the research published papers in indexed medical and immunological are our references to write the review.This article provides an overview guide in immunity, and describes the properties and classification of the immune system and different types of host defense mechanisms. Focusing on auto-immune diseases, immuno-inflammatory disorders can be categorized as auto-inflammatory or auto-immune inflammasome processes that differ in the presentation of the disease. Therefore, each disease has its properties based on how the immune cells function under certain conditions that result in the cell output becoming abnormal.

KEY WORDS: IMMUNE, DISEASES, IMMUNOLOGY, LEUCOCYTES, SYSTEM, INNATE, CYTOKINES..

INTRODUCTION

The immune system is the most important system that reinforces the immunity and saves humans from all infections, which use a large number of immune cells to function successfully. Immunity operates in a coordinated manner to tackle multiple environmental risks. In order to produce an immune response against disease and certain types of cancer, an adaptive immune system can be activated. The adaptive immune system protects the body against certain external invaders through T and B cell receptors on T and B lymphocyte surfaces (Chaplin 2010). As a result of the recombination of gene fragments, these receptors capable of recognizing different antigens. With contrast, the innate immune response is not antigen-specific, and molecular patterns of a possible future pathogen can be detected using innate immune cells and their mediators (Nicholson,

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2016).Bone marrow is primary hematopoietic organ which are responsible for preservation and continuously renewed and differentiation into mature white blood cells (Koliaraki et al., 2020).

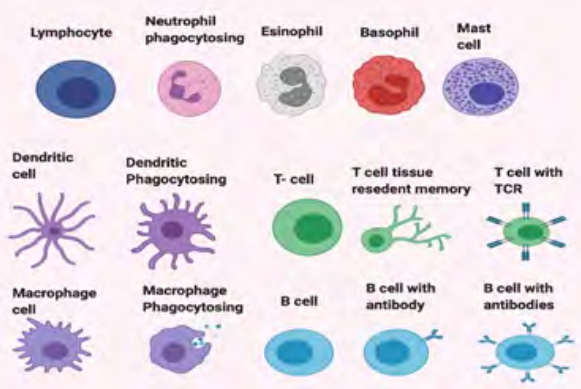
Some of them then undergo important high-school training before being released to patrol the body. Five different white blood cell types will be derived into the bloodstream (Table 1). These population groups can be further subdivided on the basis of proteins expressed in their cell membranes by an immunologist or hematologist. On their surface, the cells express several hundred different receptor forms that can bind to soluble molecules like cytokines. Pattern recognition receptors (PRR) such as duty-like receptors track molecular pathogen patterns and induce specific signaling pathways to trigger the immune system response (Lee, 2016).The natural immunity generated after the first encounter and resists of a particular species of microorganisms. The immunizations of dead or life microorganisms will trigger acquired (adaptive or specific) immunity for the individual. However, acquired immunity can occur in a natural or artificial way (Varadé et al., 2020).

Immune cells: The role of various types of white blood cells is fighting infections. The most common forms of these are cell macrophages and neutrophils of the innate immune system (Figure 1). Macrophages are a specific type involved in the detection. Both macrophages and neutrophils respond rapidly to infectious pathogenic agents producing cytokines that recruit and activate immune cells. They secrete enzymes and chemicals that digest protein. Then they swallow up and digest the damage, a process that is called phagocytosis. Such cells embody adaptation and memory functions, which enable the immune system to respond increasingly specifically and remember individual infection types (Prame et al., 2018).

Natural killer cells (NK) play an important role in managing viral infections and cancers as well as in the regulation of macrophages and dendritic cells(Table 1). Granulocyte cells (basophils, Mast cells, and eosinophils) are able to protect the human body from parasites and modulate immune responses(Brodin, 2017). Furthermore, B lymphocytes' functional role is the production of "antibody (Ab)" as an effector tool, which is an immunoglobulin molecule biochemically. Memory B cells are key to quick evelopment after re-infection with protective immunity(Laidlaw and Cyster 2020). T lymphocytes are divided into two subclasses according to their surface markers: CD4 and CD8 lymphocytes (Karim et al 2015).

Throughout innate and adaptive crosstalk, dendritic cells demonstrate a key role in the initiation of adaptive immune responses to pathogens (Waisman et al., 2017). Mast cells able to support vascular permeability and accelerate leukocyte recruitment by inducing pro-inflammatory mediators within the immune system (da Silva et al ., 2014). The natural killer cells are similar in terms of morphology with the lymphocytes except that the cytoplasm has granules. Natural killer cells are part of the innate immune system and help in defend the host by killing the foreign pathogen (Bald et al., 2020).

Figure 1: Cells of the immune system.



Of the same progenitors natural killer cells evolve but vary into separate sub-sets that vary in development of cytokine, cytotoxicity, homing, and memory (Collins et al., 2019). It was found that, there is a correlation between NK cells and genes that encode immune checkpoint proteins (Wu et al., 2019). Macrophages, B lymphocytes, and dendritic cells are antigen-presenting cells (APC) that can present antigen. Dendritic cell phagocytosis step is the connection point between the two types of the immune system (adaptive and innate), which present the essential dendritic cells in the human body(de Jong et al., 2006).The provision of pathogen defensive immune responses involves unique niches wherein leucocytes are trained by numerous types of cells, including mesenchymal cells (Koliaraki et al., 2020).

Immunoglobulins: Immunoglobulins are glycoprotein molecules made from plasma cells, also known as antibodies. The first antigen-specific receptor to be identified is immunoglobulin which is usually drawn as a cartoon in the shape of a Y (Table 2). Two identical light and heavy chains are present in the Y shape. Antibodies bind strongly on a target (antigen) and signal immune cells (Figure 2). There are two physical types of antibodies: a soluble form that is secreted from cells free of plasma in the blood. In addition to membrane-bound form attached to the cell surface called the B-cell receptor. There are three effector functions of Abs: neutralization, opsonization, and complement fixation. Nowadays, there are five classes of Ig found in the human body, termed as IgA, IgG, IgM, IgE, IgD (Table 2) (Janeway et al., 2001, Senger et al., 2015).

IgG is the largest monomer circulating antibodies, accounting for 80% and 75% of the total serum antibodies. IgG provides the bulk of pathogens-based antibody immunity.

IgA is a dimeric antibody found in mucous secretions as well as respiratory, genitourinary, gastrointestinal secretions.

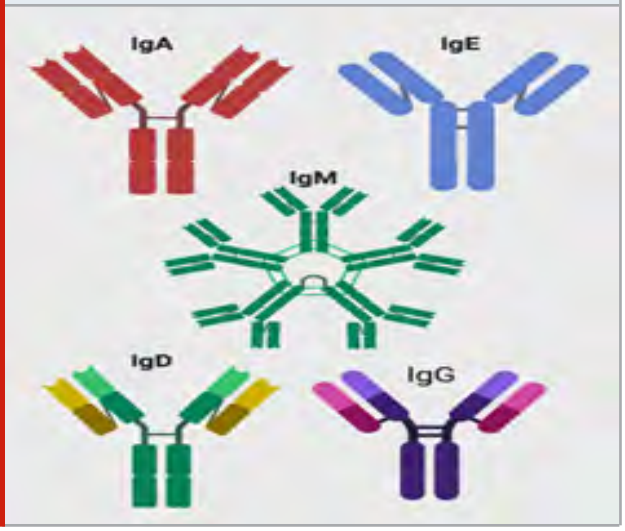
IgM is the largest antibody with pentamer structures. It is expressed on the surface of B lymphocyte and can also found in serum.
IgD is monovalent and is found on the B-lymphocyte surface that serves as an antigen receptor for activating B cells in combination with the monomeric IgM.
IgE is a monomeric antibody and usually bound to tissue cells, mostly mast cells and associated with the allergic response.

Adaptive immunity: Adaptive immunity has evolved to give both self and non-self-antigens a broader and finer repertoire of recognition. Immunoglobulins (Ig) or antibodies are plasma-cell glycoproteins instructed by specific immunogens (Sotiropoulos and Tsihrintzis 2017). The immunogen interacts with the B cell receptor (BCR) on the cell surface and produces a signal to induce the synthesis of antibody which are highly specific to immunogen that stimulated the B cell. However, the immune system remembers the antigens due to the growth of memory B cells, which triggered a previous reaction as a memory (Bonilla and Oettgen, 2010).

Table 1. White blood cells and their function (Handin et al 2003).

White blood cell	Appearance	Nucleus	Diameter	Function
Neutrophils		Multilobed	10–12 µ	Early responder Phgocytosis Local killing
Lynmphocytes		Deeply staining, eccentric	-Small lymphocytes 7–8 µ -Large lymphocytes12–15 µ	Adaptive immunity Sub-divided into T-cells and B-cells
Monocytes		Kidney shaped	15–30 µ	Early responder Phgocytosis Mature as macrophage in the tissue
Basophil		Bi-lobed or tri-lobed	12–15 µ	Bind IgE Allergy Defense against parasites
Eosinophil		Bi-lobed	10–12 µ	Bind IgE Allergy Defense against parasites

Figure 2: The diversity of immunoglobulin types.



Adaptive immunity involves tightly regulation between T and B cells, which facilitate pathogenic pathways of immune effectors, immunological memory generation and the control of host immune homeostasis (McComb et al., 2013).The T-cell function of the immune response is primarily involved in the recognition and removal of infected cells. In addition, T cells can identify antigen peptide fragments that engaged by APC via a phagocytosis or pinocytosis process. The αβ TCR surface expression is the dominant class of T cells. These receptors are primarily developed to identify peptide antigens in a complex comprising MHC proteins of class I or class II (Lee 2019).

Innate immunity: Innate signals are necessary to activate the adaptive immune system. The adaptive immune system takes advantage of an innate system's ability to discriminate between contact with dangerous pathogens and safety or even useful microbes and environmental factors by using innate signals to help initiate its reactions. The innate and adaptive divisions of the immune response should, therefore, be seen as complementary and collaborative (Chaplin 2010). Innate and adaptive system work side by side as a team against invading microbes, one establish to identify and the other one recognizes and remember specific pathogens. These depend on a group of proteins and phagocytic cells, which recognize and become quickly activated for the destruction of invaders by preserved pathogen characteristics, (Netea et al., 2020).

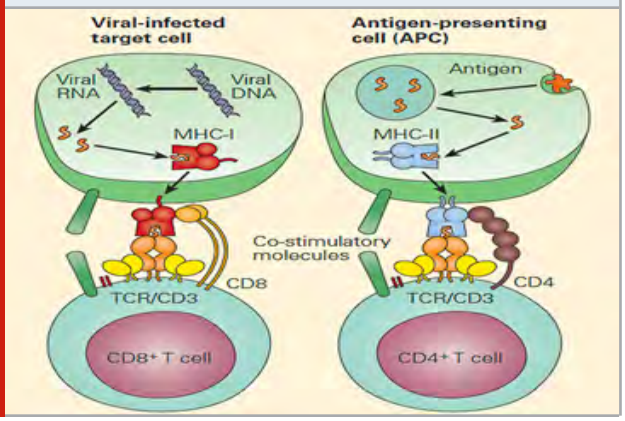
Major Histocompatibility Molecules: The infected cell used to produce future offspring microbes must be

detected and destroyed. An important role of the immune response of T cells is the detection and destruction of damaged cells. Moreover, T cells able to identify peptide antigen fragments that were absorbed by APC during phagocytosis or pinocytosis. The identification of damage host cells will be completed using an auto-component and a microbial structure recognized by T cells (Tomar and De 2014).The MHC family of molecules is the elegant way to solve both an auto-structure and a microbial determinant. MHC molecules are cell surface glycoproteins that attach to peptide-protein fragments. Peptides either synthesized in the cell (class I MHC molecules) or absorbed by the cell and then proteolytically processed (Class II MHC molecules) (figure 3) (Godfrey et al., 2004; Wieczorek et al.2017).

Table 2. The 5 major classes of immunoglobulin types which have different roles in the immune response (Schroeder& Cavacini 2010 and Vidarsson et al 2014).

Antibody	Secreted form	Percentage in serum	Cross placenta	No. of antigen binding sits	Function
IgA	Dimer	13 %	No	4	Mucosal immunity
IgE	Monomer	0.002 %	No	2	Defense against parasites Allergy
IgG	Monomer	80 %	Yes	2	Complement activation Neonatal immunity
IgM	Pentamer	6 %	No	10	Native B-cell receptor Natural antibodies Complement activation
IgD	Monomer	1 %	No	2	Native B-cell receptor

Figure 3: CD8 T cells usually recognize the endogenous antigens (left panel), CD4 T cells usually recognize the exogenous antigens (right panel) (Bonini 2012)



MHC I molecules are glycoproteins located on all nucleated cells surface. Their function is to demonstrate peptide fragments within the cell to cytotoxic T cells (CD8+ T Cells). This step will trigger immediate immune system reactions to a specific antigen present with the aid of an I protein in MHC I (Blander 2016). MHC II molecules are glycoproteins, which interact with antigen-presenting cells (APCs) such as dendritic and macrophages cells. APCs engulf foreign particles then present antigen to T and B cells. Phagocytosis contributes to the epitope loading process within the MCH Class II molecule; endocytosis is used to digest extract proteins from lysosomes. Epitopic peptide fragments that arise from this progression is loaded into the MHC Class II molecules before they leave to the cell surface (Rock et al., 2016). Exogenous antigens which initiated extracellularly from foreign organisms such as bacteria will easily recognize by helper T cell lymphocytes (CD4+ T cells) inducing antibody production and attracts immune cells to the infection region (Aluri et al., 2018).

Complements: The complement structure is a significant element within the immunity system. The complement system is prepared by a wide range of separate plasma proteins, reacting with pathogens to induce a variety of inflammatory reactions that serve to combat infection and improve their ability to eliminate microbes and damaged cells from body system, (Carroll 2004). The complements activation process follows several different ways; 1)classical pathway which is activated by direct attachment between complement component C1q and the pathogen surface, or using an antibody. 2) the MB-lectin pathway which is produced by mannan-binding lectin, 3) the alternative pathway that activated directly on pathogen surfaces. All of these pathways generate a crucial enzymatic activity that, in turn, generates the effector molecules of complement. The three key effects of complement activation include pathogens ' opsonization, inflammatory cell recruitment and direct killing of pathogens (Spiering 2015 & Kolev and Markiewski 2018).

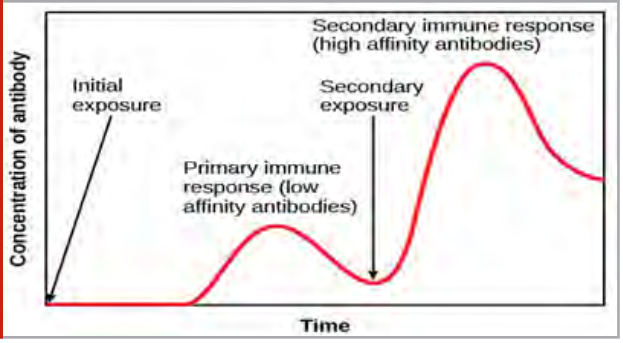
Cytokines: Cytokines are cell-released proteins, which have a specific effect on cell-to-cell interactions and communication. Pro-inflammatory and anti-inflammatory cytokines are both available. Cytokines are formed by various cell types, however, T cells (Th) and macrophages are the predominant producers. Cytokines work by binding to a receptor, which sends a signal to the receiver cell that leads to a functional or phenotype transition. Such signal cascades are complex and include

a variety of environmental considerations. There are currently many more cytokines examined as therapeutic objectives or as therapeutic agents (Seillet et al., 2014)

Toll-Like Receptors: Toll-like receptors are a protein class that shows a critical function in the innate immune system. Ten human Toll-like (TLR) receptors were identified now. They are usually expressed on main cells include macrophages and dendritic cells that identify pathogen-associated molecule patterns (PAMPs) resulting from different microbes. In many respects of the innate immune response to certain pathogens, TLR signaling tends to be divergent and plays an important role, (Kawasaki and Kawai ,2014 & Liu et al., 2020).

Immune memory: The classical immune memory assessment is based on the reaction of the antibody. Specific antibody level rises after an infection (or vaccination), then decreases over time to lower levels, not as small as in a naive state. Recharging the same stimulus results in faster development of more antibodies and high avidity through the evolution and selection of higher affinity antibodies (figure 4). There are also other shifts, particularly since the prevalent antibody response isotype would typically have shifted to IgG (and IgA or IgE) (Paul 2015).

Figure 4: Primary response shows the initial production of antibodies from plasma cells. Memory cells differentiate to plasma cells after the same microbe will expose. Differentiated plasma cells induce antibodies that will continuously discharge a large number of antibodies (Paul 2015).



Immune system diseases: Immunological disorders are conditions caused by an immune system deficiency or heterogeneous aberrations that cause the system to work against itself. In fact, this condition may occur due to an irregular activation of the immune system because of a regulatory function weakening that has damaged it and triggers a strong reaction to it. Such diseases also serve as one of the world's leading deaths. (Aribi, 2017). Autoimmunity is conceptually considered as a defect of the lymphocyte selection, either in B or T, with aberrant lymphocytic responses. In recent years the autoimmunity hypothesis has been renewed by an improved genetic understanding of both common and rare diseases, coupled with mutations that represent disturbances of the immune system, ranging from Thyme, B, and T to T regulatory cells (Dosanjh 2015).

Self and Non-self Discrimination: Self-tolerance is called the capacity of the immune response to prevent damaging self-tissues. Therefore, the wide variety of autoimmune diseases underlie self-tolerance failure (Furusawa and Yamaguchi 2016). As a significant aspect of the immune system's T-cell arm is to detect cells affected by viruses, intracellular bacteria or other intracellular parasites, T-cells can form an advance mechanism that identifies foreign antigens as a complex molecule. T cells are responsible to spot and discover both self-structuring and external antigens when they preserve their self-tolerant (Chaplin and David 2009).

Classifications and properties of immunological diseases: All immunological diseases can appear as a result of purely Autoinflammatory, autoimmune, or a combination of Autoinflammatory–autoimmune mechanisms that interact variably in the expression of disease (McGonagle & McDermott, 2006).The most common immunological diseases (Health, 2003) classified as:

Allergic Diseases: Allergic diseases can form as a result of the response of the immune system to an inappropriate alarm, which spots specific materials such as house dust and grass pollen as very harmful elements to an allergic individual.

Autoimmune Diseases: In recent years, substantial progress has been made towards learning how nearly any part of the immune system leads to systemic autoimmunity, (Tsokos 2020). The specific causes of the autoimmune disease are not exactly known, but this type of disease is appeared when the immune system cells are directed against the body cells and organs due to a break down in the immune system's recognition apparatus. When a break down happens, the body starts manufacturing auto-antibodies and Misguided T cells. T lymphocyte is the most responsible cells to different diseases such as diabetes, rheumatoid arthritis, systemic lupus erythematosus (SLE). Multiple factors are playing a role in the autoimmune disease that includes certain drugs, viruses, and sunlight. Moreover, Hormones and Heredity seem to have a role in this disease too (Wang et al., 2015).

Immune Complex Diseases: These diseases can develop when Immune complexes trapped in skin tissue, the lungs, joints, the kidneys, or blood vessels. These complexes are a Collection of interlocking antigens. Usually, they quickly removed from the bloodstreams but sometimes they continue to circulate so they trapped in the body tissues. Moreover, these complexes generate several reactions that lead to tissue damage and inflammation. Additionally, these complexes are having a role in many diseases such as viral hepatitis, malaria, and many autoimmune diseases (Carter 1973 & Clarke et al., 2018).

Immunodeficiency Disorders: The human can gain immunodeficiency disorder if the immune system misses one or more of its normal components. Usually, they can be inherited, produced unintentionally by drugs, or

acquired through infection. Moreover, immunodeficiency disorder has a role in many diseases such as severe combined immunodeficiency disease (SCID) and AIDS (Justiz 2020).

Auto-inflammation: The inflammasome and intracytoplasmic structure able to produce many proteins when the cell naturally threated, which is considered as an essential sensor. The inflammasome sub-units come together and activate an enzyme that then releases cytokines that encourage inflammation. Such cytokines can cause disease and fever once in circulation. Inflammasome activation is triggered by changes in the environment that cause the subunits to assemble (Libby 2007).

Cancers of the Immune System: This cancer can gain because of the uncontrollable growth of the immune cells such as the uncontrolled growth of leukocytes and antibody-producing plasma cells. On the other hand, this uncontrolled growth of immune system cells can lead to many different kinds of cancers such as leukemia and the cancer of the lymphoid organs (Gonzalez et al., 2018).

Evolution of immune systems: Evolution of immunity takes place at various stages during the lifespan of the host and the evolution of variants of pathogens and the shifts in frequency of lymphocyte clones throughout a person's infection (Kaufman 2010).Particular cell types are related to multiple conditions and they represent the specific body defense role of that cell type. Neonates have usually a high white blood cell count, which is eventually lowered to adult levels. An exception are the low count of Lymphocytes at birth, which in the first four years of life hits its maximum level and then eventually declines to healthy levels of adults (Katharina et al., 2015). Practically all species provide at least one type of protection to deter organisms that cause diseases. Advanced vertebrate animals, a human group, protect themselves against these microorganisms by the immune system through a complex set of defense responses. This defensive system emerged by basic defense mechanisms, although it is not completely certain which evolutionary twists and turns contributed to its development, (Broecker and Moelling 2019).

CONCLUSION

This review has been focused in particular on the immune system and immunological diseases as well as properties, classifications, and evolution of immunological diseases and immune systems. This review has the potential to shed light on the different roles of each cell in the immunity system and how this system plays an essential role in immunological diseases. Starting with the significant role of an immune system and the human body, ending with the immunological diseases and how they affect this system. The immune system is a complex network of cells, proteins, and signaling that prevent infection in the body. There are many mechanisms in the immune system to combat microbial infection. The mechanisms work together to eliminate many harmful effects and to alter the reaction to specific invading pathogens within the immune response.

The formation of immune memory is required to learn and record different pathogens that contribute to the body system to produce successful and rapid immune responses after subsequent exposure to the same pathogens. Immune system defects can lead to allergy, asthma, immune deficiency and any other autoimmune disorders. The response of the immune cells at these conditions determine the type of disease. In addition, the factors that can have a role in immunological diseases such as heredity, unintentional production by drugs, or the acquisition through infection can cause an imbalance in immunity. Immunological diseases and immunity require more future work and research to suppress and prevent the development of these types of diseases. Understanding the relations between the various immune-effector pathways will allow researchers to discover strong and effector treatment for immunomodulation, in addition, to improve vaccine development.

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Functional Constipation Due to Psychological Trauma: A Case Report with Literature Review

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ABSTRACT

Constipation is a common disorder worldwide, irrespective of age, sex, socioeconomic status, and ethnic background. Constipation adversely affects quality of life and consumes health system resources. It occurs as congenital disease (Hieschsprung disease) or as a result of secondary causes)bowel obstruction) or as a functional, medically unexplained, cause. Functional constipation (FC), also known as functional megacolon, is constipation that is not of an organic, anatomical, or iatrogenic origin. In this case report, we have searched the literature using functional, psychogenic, megacolon, constipation, functional megacolon, functional constipation as key words without any time limits. Then, titles were screened for inclusion in this paper. The evidence discussing the diagnosis and treatment approaches is bounded with no updated articles could be found. Specifically, Data on FC after a traumatic emotional trigger is scarce, and evidence for treatment approaches is limited. Our patient had fulfilled the criteria of ROME IV to diagnose FC. He has been treated by antidepressant medication and psychotherapy which lead to an increase in the number of times he is using the bathroom and decrease in the time spent in the toilet with better defecation outcome which according to the patient was a significant improvement. Our experience shows that limited data available about this condition although the prevalence of the disorder was constant around the world at a rate of 1% to 30%. The case we report herein adds to the diagnosis and management of FC area of knowledge. And it encourages the scientific community to have a standardized approach to manage the disorder.

KEY WORDS: CHILDHOOD PSYCHOLOGICAL TRAUMA; FUNCTIONAL CONSTIPATION; FUNCTIONAL MEGACOLON.

INTRODUCTION

Constipation adversely affects quality of life and consumes health system resources (Higgins and Johanson, 2004; Dennison et al., 2005). It is a common disorder worldwide, irrespective of age, sex, socioeconomic status, and ethnic background (Benninga et al., 2005; Mugie, Benninga and Di Lorenzo, 2011). The main features of constipation are a frequency of bowel movement of less than three per week, associated with a change in consistency in the stool and/or pain during the movement, and is usually accompanied by withholding maneuvers in young children(Lacy et al., 2016). Functional constipation (FC), also known as functional megacolon, is constipation that is not of an organic, anatomical, or iatrogenic origin (Hyams et al., 2006). The prevalence rate of functional constipation among children estimated at 0.7% to 29.6% and, in multiple region around the world, the prevalence of constipation rate among adult was reported from 2% to 35% (Van Den Berg, Benninga and Di Lorenzo, 2006; Peppas et al., 2008). Moreover, the prevalence of FC increased with age (Saps et al., 2020).

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There's two criteria to assess FC, Rome IV and an updated proposed Cuda criteria (Cuda, Gunnarsson and de Costa, 2018). Moreover, constipation might sometimes lead to encopresis, defined as soiling after the age of 4 years (Loening-Baucke, 1996). Data on FC after a traumatic emotional trigger is scarce, however, and evidence for treatment approaches is limited. Few data and little consensus are available about functional megacolon management. Up to our knowledge, we could only find two case reports discussing the effect of traumatic event on the functional constipation outcome. The case we report herein adds to this area of knowledge. Hopefully, it will lead to a more effective managing approach for people with functional constipation.

Case Report: The patient provided consent for publication of the case report.

Presentation: A 21-year-old man sought out-patient consultation at our clinic for chronic constipation which prevented him from attending his classes at the university. The patient had previously been admitted at a local tertiary center for urgent bowel evacuation under general anesthesia. His chief complaint at the time of consultation at our clinic was his inability to pass stool, despite normal findings on colonoscopy, colon biopsy (for exclusion of Hirschsprung's Disease), computed tomography (CT), and magnetic resonance imaging (MRI). Abdominal CT performed almost 1 year before his consultation showed a marked dilation of the large bowel loops, mainly the sigmoid and descending colon, containing fecal matter with a maximum diameter of 14.6 cm in the sigmoid colon. The patient did not report associated symptoms, such as pain or bloating, that would be suggestive of a diagnosis of irritable bowel syndrome (IBS). His laboratory investigations were all within normal range.

With regard to his family and social history, he was a first-born child and a brother to four younger sisters. He had no family history of mental disorders. He denied any current or past substance use and reported no comorbid medical illness. On presentation, he was of average body build and was well dressed and appropriately groomed for the visit. During the psychiatric interview, the patient made limited eye contact initially. He was cooperative during the interview and provided relevant coherent answers to each question. There was no evidence of a thought disorder or abnormal perception.

Relevant past history: With regard to his past history of constipation, the patient indicated that bowel training was successfully achieved during his childhood, which further excluded the diagnosis of Hirschsprung's Disease. His symptoms began around the age of 6 years. Our patient acknowledged living multiple adverse traumatic childhood events. The psychological trauma was repetitive, with multiple events each week. Physical confrontations were not uncommon in his household. He had witnessed domestic abuse on multiple occasions and for many years towards his mother. On one occasion, and while he was going to use the bathroom with an urge to defecate, he observed his parents fighting, he went back to his room and according to him, he could never pass stool spontaneously ever since. He reported seeing 'a lot of blood' when he woke up for school the next morning as a result of physical fighting between his parents. One distinctive incident for him was when his mother had sprayed gasoline and threatened the father that she would set fire to the home.

Not being able to defecate has led to soiling of his bed overnight, which occurred once or twice per week. This finding was corroborated by his aunt. He has continued to withhold urges and not to pass stool voluntarily for the past 15 years, to the point of developing abdominal distention. While taking his history, the patient confirmed that he felt an urge to defecate when away from home or when he was in a good mood. but still was unable to have a bowel movement even under these conditions. He first sought medical help for his constipation, with his aunt, at the age of 8 years. At that time, he was treated using laxatives. According to him, this treatment was ineffective; however, he did not return for follow-up over the subsequent 3 years due to his chaotic family situation. He persisted with the use of laxatives up to 2 years prior to the current consultation.

His parents got separated when he was 16-year-old, and he went to live with his aunt at that time. She enabled him to follow-up with medical care, including a thorough investigation of his constipation. Physical examinations and investigations previously performed after encopresis developed were repeated. All results were normal. The patient subsequently presented to psychiatry for assessment. At the first assessment, 4 years prior to the current consultation, the patient was prescribed psychotropic medication for a period of 2 months. According to him, his condition improved, and he felt the urge to evacuate. Unfortunately, his family refused psychiatric medications, probably due to the stigma, and the medication was discontinued, with chronic constipation persisting.

This year, at the age of 21 years, he started to use diapers as a measure to avoid encopresis accidents and allow him to attend university classes. He presented to our service alone and chose to follow-up with psychiatry. At the time of the consult, he was a foundation year student at university and had accumulated multiple course failure over the last year. Along the course of his illness, the patient reported having major depressive episodes and occasional panic attacks. He had sustained a sexual abuse incident at the age of 12 years and reported symptoms

of post-traumatic stress disorder. He had planned suicide twice during his illness but did not follow through due to his religious beliefs.

Treatment: The patient was treated using escitalopram (10 mg, daily) combined with psychotherapy. After three follow-up visits (2 weeks apart), he reported an overall improvement in his psychological well-being. He had developed future plans and was enjoying life more than before. At the fourth visit, the patient was introduced to the biofeedback approach, including an explanation of the elimination process and of the benefit of sitting on a toilet for 10 minutes after each meal.

Follow-up and outcome: For the first 6 months after the start of his medication, the patient was very compliant with his daily dosing of escitalopram and showed a good treatment response. He reported improvement in his overall psychological well-beings and was able to attend classes and started to get into a routine. He also reported an improvement in his overall sense of using the toilet, with only mild anxiety. However, the patient missed his 6-month follow-up appointment (4-weeks after the start of biofeedback therapy). Contacted by phone, he confirmed continued progress in the aforementioned improvement. With regard to the biofeedback approach, he had been adherent to the prescribed sitting on the toilet for 10 min after each meal, reporting that he had the feeling of passing stool after the third trial.

However, he did not proceed as he was afraid of painful defecation. As he had missed his follow-up appointment, he ran out of antidepressant medication. Family opposition to use of psychotropic medications made him unable to refill his prescription. Moreover, as his family chaos was persisting, he reported a major depressive episode. He was still using diapers and was missing numerous classes. He did return for follow-up and has been taking escitalopram for two months, reporting that he is doing very well, without side effects. He is able to use the washroom 4-5 times per week. He reported spending 4 minutes in average on toilet. Although most of the defecation attempts fail and occasionally successful defecations were incomplete, he was satisfied. He is now aware of the effect of being anxious and how his mood increasing the tension in the pelvic floor muscles. These outcomes can be compared to the pre-treatment data where the patient was never using the toilet.

DISCUSSION

In a systematic review, the mean prevalence of FC was reported between 0.7% to 29.6% with a median of 8.9% of children, with a conflicting data about the gender ratio. However, the majority of the reviewed articles reported a female predominance in the prevalence of constipation(Van Den Berg, Benninga and Di Lorenzo, 2006; Cuda, Gunnarsson and de Costa, 2018).3 The prevalence rate was almost duplicated in another systematic review in 2018 (Koppen et al., 2018). Carson et al has reported that among neurology outpatient visitors complaining of constipation, FC was more prevalent than organic constipation (Carson et al., 2014). The prevalence rate of FC seems to be increased with age (Saps et al., 2020).

Of all reported cases of FC, however, we identified only one case linking psychological trauma to the development of FC, and another reported an exacerbation of FC symptoms following a psychological trauma (Brody, 1963; McGuire, Rothernbergh and Tyler, 1983). I quote from Brody, 1963 the following: "Nina had reacted with psychogenic withdrawal and an exacerbation of an earlier pattern of soiling and bowel retention". Up to our knowledge, no recent study could be found in the literature linking the psychological wellbeing of the patient with the emergent of the symptoms. However, psychological wellbeing was shown to be affected by FC (Vriesman et al., 2019).

Table 1. ROME IV criteria adopted from the study published by (Lacy et al., 2016)

Diagnostic criteriaa for functional constipation
1-Must include 2 or more of the following:[b]
a. Straining during more than one-fourth (25%) of the defecation events.
b. Lumpy or hard stools (BSFS 1-2) in more than one-fourth (25%) of the defecation events.
c. Sensation of incomplete evacuation in more than one-fourth (25%) of the defecation events.
d. Sensation of anorectal obstruction/blockage in more than one-fourth (25%) of the defecation events.
e. Manual maneuvers to facilitate more than one-fourth (25%) of the defecation events (e.g., digital evacuation, support of the pelvic floor).
f. Fewer than 3 spontaneous bowel movements per week.
2- Loose stools are rarely passed without the use of laxatives.
3- Insufficient criteria for irritable bowel syndrome

a Criteria fulfilled for the last 3 months, with symptom onset at least 6 months prior to diagnosis.
b For research studies, patients meeting the criteria for opioid-induced constipation should not be diagnosed with functional constipation (FC) as these two conditions may overlap. Clinicians should be aware of the possibility of this overlap.

Treating patients presenting with FC as a case of Irritable Bowel Syndrome (IBS) is not uncommon (Tosto et al., 2020). Interestingly, IBS has been proposed to be classified as a medically unexplained or functional disorder (Wessely, Nimnuan and Sharpe, 1999). However, the diagnostic criteria of FC remain obscure. We could find two criteria ROME IV (Lacy and Patel, 2017) and a proposed newer criteria suggested by Cuda (Cuda, Gunnarsson and de Costa, 2018) to assess the diagnosis. According to ROME IV criteria, FC is primarily differentiated from IBS by the relief of abdominal pain after defecation(see table 1)(Lacy and Patel, 2017).

In our patient, abdominal pain or bloating were not predominant symptoms. Additionally, he complained of abdominal distention related to constipation, rather than a sense of bloating which is usually a feature of IBS Rome criteria and a diagnostic approach to irritable bowel syndrome(Lacy and Patel, 2017), and repeated incidences of soiling and fecal incontinence due to the stool impaction that affected his quality of life. However, although IBS was excluded, he has been treated as a case of IBS during the 15 years of suffering. Treatment used for IBS, such as the use of laxatives or enemas (Dalrymple and Bullock, 2008), were not effective on the long-term as it occasionally provided a symptomatic relief . Psychological stress might cause an exacerbation in IBS symptoms (Qin et al., 2014). Similarly, to FC, different studies reported an increase in psychological distress and decreased well-being with FC, as previously reported in functional neurological disorders (Merkel et al., 1993; Glia and Lindberg, 1997; Keynejad et al., 2018).

Another resemblance between functional neurological disorder (i.e. medically unexplained symptoms) and FC is the predominance of female to male patients(Higgins and Johanson, 2004; Carson and Lehn, 2016). Childhood adversity, like in this case, is a well-recognized risk factor for the development of functional neurological symptoms (Katon, Sullivan and Walker, 2001). The diagnostic criteria for FC have been presented by Hyams and his colleagues with the ROME IV criteria widely accepted (Hyams et al., 2016). Our patient did, however, fulfill the ROME IV criteria of FC. The aforementioned management plan that was used for our patient was similar to the approaches used for functional neurological symptoms has yield to a significant improvement (Wessely, Nimnuan and Sharpe, 1999; Stone, Carson and Sharpe, 2005; Carson et al., 2014).

CONCLUSION

Previous cases have reported psychological trauma as a contributing factor to functional megacolon. Further accumulation of cases is warranted to determine the appropriate treatment approach to improve the physical health, and psychological well-being of patients with FC. Functional constipation might be considered as a specifier in the Diagnostic and Statistical Manual of Mental Disorders (DSM) for the functional neurological symptoms, along with sensory and motor specifier that are already included. This, as well, may shed light on the importance of evaluating psychological trauma in any individual presenting with FC. We recognize the limitation of a case report in the hierarchy of evidence and there might be a recall bias. Therefore, we recommend a population-based study to determine the prevalence of chronic constipation in children and adults and to determine associated factors to inform treatment.

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Rheumatoid Arthritis and Comorbid Conditions; Is There A Missing link?

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ABSTRACT

Rheumatoid arthritis (RA) is a joint autoimmune disorder with unknown aetiology, affecting 1% of the world's population causing permanent deformation of the affected joint. The main objective of this study was to investigate the prevalence of comorbidities in a population-based cohort of individuals suffering from rheumatoid arthritis (RA) as compared to normal population. Data of 877 individuals with 402 RA and 475 Age- and sex- matched controls without RA were taken with their consent from the population of Gujarat. As compared to the controls, most of the investigated comorbidities were more frequent in the individuals with RA. On further dividing them into five different regions within Gujarat based on their origin-it was found that both in the RA and the control population belonging to North Gujarat were maximum and least sample population was belonging to Kutch region. Severe Anaemia, fatigue and depression were found more in RA than the control population. It was found that in addition to RA, some other disease conditions like Hypertension, Diabetes, Kidney problems, thyroid dysfunction, cholesterol and breathing problems were found in 40.55% of the RA population and 11.58% of the control population. BMI results also showed maximum obesity in RA population as compared to the control population in both genders (73.63% vs 34.74). Females were found to be more prone than the males in most of the comorbidity. Compared to the control population, persons with RA present with increased prevalence of numerous comorbidities. Patients with RA and multimorbidity are at risk of insufficient rheumatologically care and poorer patient-reported outcomes. Thus, a proper understanding of different comorbid condition along with RA can improve the quality of life as well as decrease the mortality rate of the RA patients.

KEY WORDS: COMORBID CONDITIONS, GUJARAT, RHEUMATOID ARTHRITIS.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic disease that affects the joints, connective tissues, muscles, tendons and fibrous tissue. The prevalence of RA is estimated to be ~1% of the population worldwide and is more common in women. The prevalence of RA in India is 0.75% (Malaviya et al.,1993; Handa et al., 2016). Although the aetiology and pathogenic mechanism underlying the development of RA remain unclear, the combination of a susceptible genetic background interplaying with environmental factors has been considered to be associated with the development of this complex disorder. It is well established that there are several risk factors like age, gender, hormonal levels, alcohol, cigarette smoking, socioeconomic status, and dietary habits that is contributing to the initiation and promotion of this complex disorder.

When RA is left uncontrolled, the RA patient may experience joint deterioration, severe disability, decreased

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quality of life, the onset of comorbidities and premature mortality (Choy and Panayi, 2001; Handa et al., 2016). It has been observed that RA comes with one or more comorbid conditions. Comorbid conditions can be broadly arisen by - (i) arising due to disease pathology and (ii) due to treatment drugs used. The most affected organs includes eyes, heart, lungs, bones and also psychological disorders are commonly found in addition to RA, (Wolfe et al., 1994 ; Gonzalez et al., 2007; Salliot and van der Heijde, 2008; Emamifar and Hansen, 2016; Singh et al., 2016; Levytskyi et al., 2019).

More comorbid conditions have been seen to be leading to a higher death rate in RA (S E Gabriel, 2008). Moreover other risk factors like smoking might be playing a role in causing comorbidity (Liao and Solomon, 2013). Comorbidity in addition to RA is an economic burden on patients, their families, and society which also a challenge to the rheumatologists (Cross et al., 2014; Onna and Boonen, 2016). Although prevalence of comorbidities is more common in RA than controls, comorbidity is many a times under recognized and undertreated (MacLean et al., 2000; Dougados et al., 2015; Mohan et al., 2017). There are many studies showing comorbidities in RA but some studies lack a comparison with the non-RA population. Moreover, the data regarding medical comorbidities associated with RA in previous studies were mainly from western populations. So, the aim of this study was to determine the prevalence of comorbidities in adult population with RA along with non-RA population of Gujarat, India.

MATERIAL AND METHODS

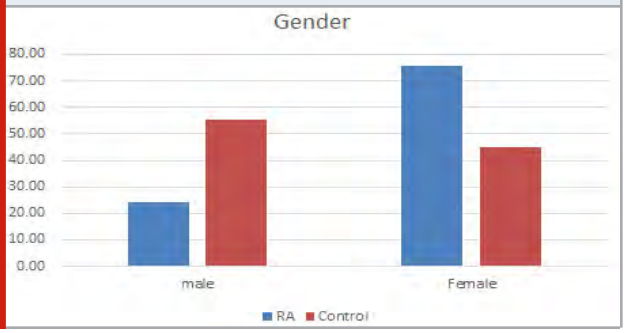
Total of 402 RA patients classified according to American college of Rheumatology 2010 criteria (Aletaha et al., 2010). Retrospective analysis of the patients attending the rheumatology outpatient clinic between the years December 2014 to December 2015 were analyzed. A detailed questionnaire regarding their pervious health history and other details as well was taken. Prior to participation, the purpose of the study was explained to all the subjects and their informed consent was taken. A total of 475 ethnically matched unrelated healthy volunteers with a negative history of any joint disorders were taken up as control population for the study. Hemoglobin levels were measured using the Sahli's method.

RESULTS AND DISCUSSION

It is a well-established fact that the inflammation in RA is also affecting various organs like the bone, lungs, cardiovascular system and many more apart from the joints leading to excess mortality (S E Gabriel, 2008; Gabriel and Michaud, 2009; Dougados et al., 2014).

1.Total population: Total samples collected were 877 out of which 402 were of RA patients and 475 samples belonged to the control population. Out of which 98 (24.38%) were found to be male and 304 (75.62%) were females in the diseased population whereas in the control population 262 (55.16%) were males and 213 (44.84%) were females (Figure 1).

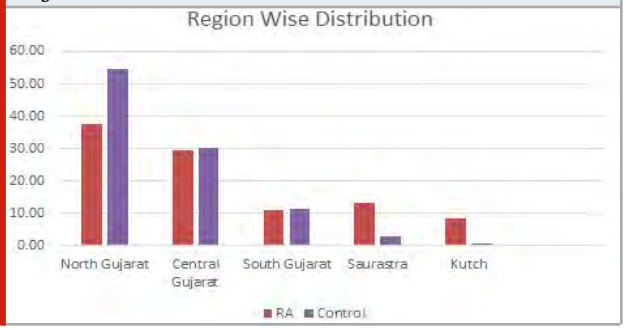
Figure 1: Prevalence of RA in males and females



Supporting previous studies, females were found to be approximately 3 times more prone to the disease than the males (Van Vollenhoven, 2009; Patel, 2011).

2. Region wise distribution: The samples were further divided into five different regions within Gujarat based on their origin which are- North Gujarat, Central Gujarat, South Gujarat, Saurastra and Kutch. It was found that both in the RA and the control population belonging to North Gujarat were maximum with 37.56% and 54.53% respectively. Central Gujarat was consisting of 29.60% RA population and 30.32% of the control population. This was followed by South Gujarat with almost same amount of population in both RA and control population with 11.19% and 11.58% respectively. In Saurastra, RA population was 13.18% and control population was on 2.95%. Least sample population was belonging to Kutch region with 8.40% in RA and 0.63% in the control population as shown in figure 2.

Figure 2: Region Wise Distribution of the population in Gujarat



3. Anaemia: It was observed that severe anaemia was found more in RA population in both the genders with males having 10.14% severe anaemia and 17.65% of severe anaemic female population as compared to the control population with no severe anaemic male population and 8.97% in the female population. Again percentage of moderated anaemia was found to be more which is 59.42% in RA male population as compared to 36.00% in the control male population. No significant change was found in the female population in RA with 62.61% and control population with 68.59%. It was

furthermore found that mild anaemia was more in the control population with 64.00% in male population and 22.44% in female population as compare to the RA population with 30.43% in male population and 18.91% in the female population. This suggests that there might be a possibility that due to RA, mild anaemia may lead to moderate or severe anaemia (Figure 3 and 4) (Goyal et al., 2018).

Figure 3: Comparison of the Anaemic population

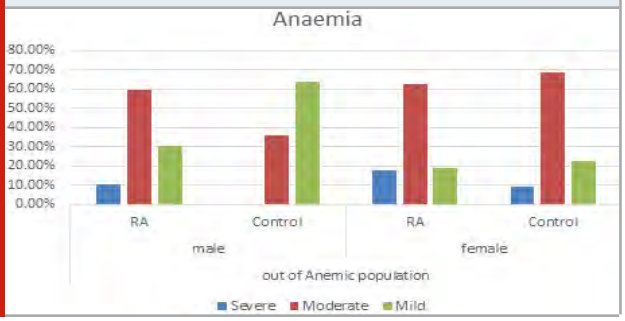
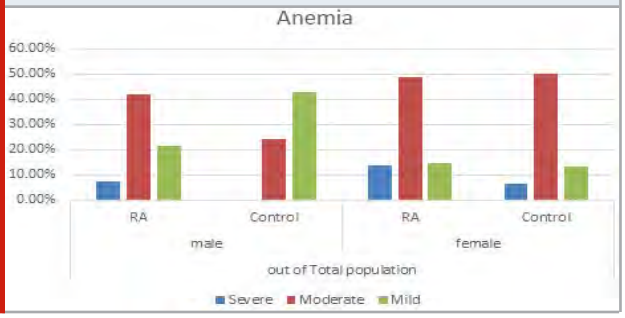


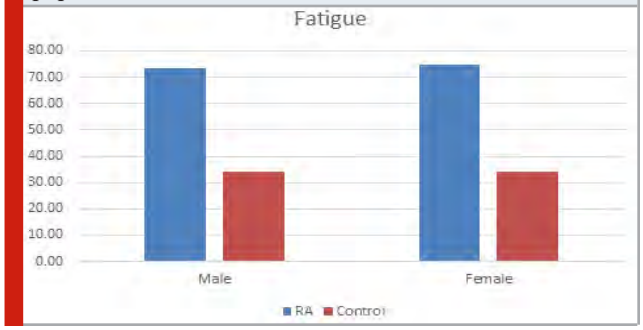
Figure 4: Distribution of Anaemia in total population



Overall anaemia of chronic disease and iron deficiency anaemia are frequent causes of anaemia in RA patients(Agrawal et al., 2006; Goyal et al., 2018). Anemia was found in 75.87% of the patient population as compared to 69.68% in the control population in our study which correlates with the previous studies of India (Baer et al., 1990; Agrawal et al., 2006). In this study, severe anaemia was found more in RA population in both the genders having severe anaemia as compared to the control population. Mild Anaemia was seen in the control population more than the diseased population suggesting that there might be a possibility that due to RA, mild anaemia may lead to moderate or severe anaemia supporting various reports implicating anaemia playing an important role in the disease activity (Goyal et al., 2018).

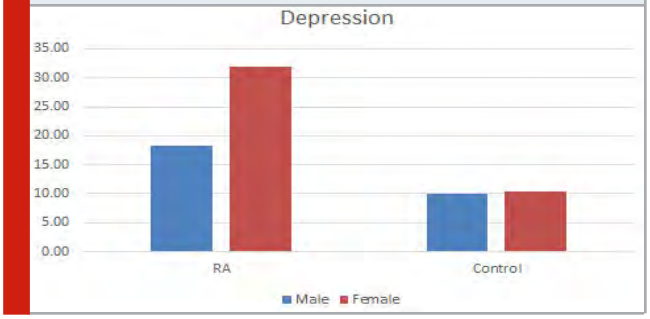
4. Fatigue: R.P. Riemsma et al. (1998) showed the prevalence of fatigue in RA population to be 80-93%. High fatigue levels are common in RA and are mainly linked to pain and depression (Riemsma, 1998). It has also been observed in one of the study by Pollard, that association with disease activity is secondary, which also correlates with our study that population of RA (74.3%) suffered more from fatigue condition than control population (34.31%) as shown in fig. 5 (Pollard et al., 2006).

Figure 5: Fatigue condition found in both the populations



5. Depression: C. Sheehy et al. (2006) mentioned the prevalence of depressive disorder in patients with RA to be ranging between 13 and 20% (Sheehy et al., 2006). Dickens et al. (2003) found 39.2% had definite symptoms of depression. And further concluded that depression was significantly more common among RA patients than healthy individuals and was influenced by the level of pain but not by demographic factors (Dickens et al., 2003). This study is supporting our study showing that 28.60% of the diseased population were suffering from depression as compared to 10.10% of the control population. Prevalence of depression was found more in the females as compared to males as shown in the figure 6. There is much argument as to whether depression simply reflects a reaction to the pain of RA or whether depression contributes to pain experience. There are studies also stating that depression and psychological stress have been shown to result in immune dysfunction (Herbert TB, 1993; Dickens and Creed, 2001). There are studies suggesting that depression also relates to poorer RA outcomes. So, it is crucial that emphasis on the detection and treatment of depression in RA to improve the lifestyle of the patients (Matcham et al., 2013).

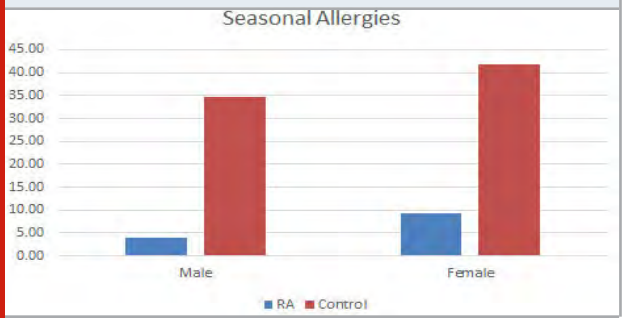
Figure 6: Level of depression found in both the populations



6. Seasonal Allergies: Surprising to find that seasonal allergies were found to be occurring more in control population than the RA population as seen in figure 7, in oppose to studies that allergy increases with RA (Karsh et al., 2005). Male control population were having 34.73% of seasonal allergies as compared to 41.78% in

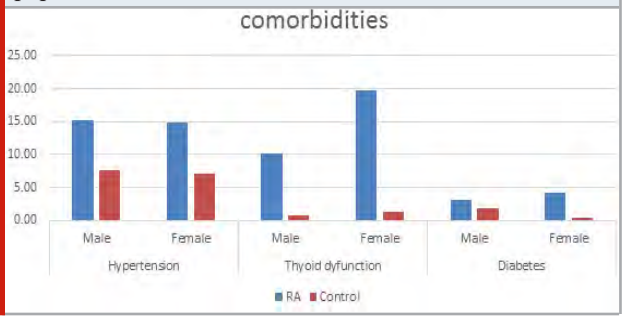
the male RA population. Also, in the female population in controls were having 41.78% of seasonal allergies as compared to 9.21% of the female RA population. There are no studies supporting this result. So, more studies in this area might broaden the understanding the correlation between the two (Matcham et al., 2013).

Figure 7: Effect of seasonal allergies found in the populations



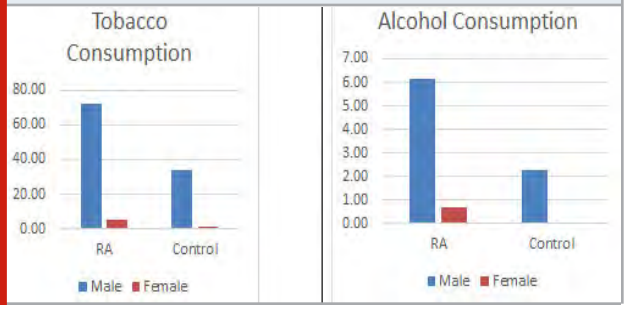
7. BMI: There have been many studies showing an association between higher body mass and many inflammatory or autoimmune disorders. Overweight or obesity measured by body mass index (BMI) corresponds to an abnormal accumulation of adipose tissue within the body, which secretes proinflammatory and anti-inflammatory metabolically and hormonally active substances, and produces cytokines and chemokines thus contributing in the processes associated with inflammation and immunity (Fantuzzi, 2005; Touyz Rhian M., 2005; Trayhurn and Wood, 2005). There are many reports stating the dominance of obesity in females and its possible role in developing RA (Symmons, 2005). Our study validates this observation with higher BMI found more in female RA population (82.09%) than the male population. The predominance in the female population is still unclear. Moreover higher BMI was also found in the disease population supporting previous studies (Versini et al., 2014; Qin et al., 2015; Albrecht et al., 2016; Hugo et al., 2017) (Figure 10).

Figure 8: Different comorbidities found in the populations



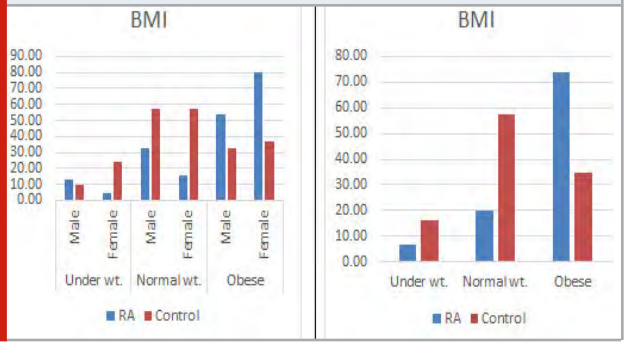
8. Co-morbidities: There are various reports showing higher death rate in patients with RA might to be the consequence of more serious co-morbid conditions (Gabriel, 2008; Gullick and Scott, 2011). Many studies have showed that comorbid conditions were between 40 and 67% of the RA population (Innala et al., 2016; Fz, 2019) . In this study, it was found that in addition to RA, some other disease conditions like hypertension, diabetes, kidney problems, thyroid dysfunction, higher cholesterol and breathing problems were prevailing in about 40% of the disease population as compared to the control population (11%) correlating with a study done by Chandrashekara, (2017) in south India. The highest comorbid condition in the RA population was found to be thyroid dysfunction (17.41%), hypertension (14.93%) followed by diabetes (3.98%) (figure 8) (Chandrashekara et al., 2017).

Figure 9: Tobacco and Alcohol consumption found in both the populations



An association between RA and thyroid dysfunction with or without autoimmune origin has been reported in 5% to 34% of patients with RA (Shiroky et al., 1993; Przygodzka and Filipowicz-Sosnowska, 2009; Jeong et al., 2017). This study supports the positive association between RA and thyroid disorder. It was found that RA population was suffering from thyroid dysfunction more as compared to control population. However, this prevalence (17.41%) in the diseased population was found to be quite less as compared to the previous studies (Atzeni et al., 2008) with 10.20% and 19.74% in male and female population respectively. Control population with 0.76% males and 1.41% females were suffering with thyroid dysfunction. The relationship between RA and thyroid dysfunction is in agreement with the other studies stating the female dominance (Deighton et al., 1992; Bianchi et al., 1993; Andonopoulos et al., 1996).

Figure 10: BMI found in the studied population



Shiroky (1993) showed the increased number of thyroid disorder in the diseased females than the similar control ones, thus supporting our results (Shiroky et al., 1993; Przygodzka and Filipowicz-Sosnowska, 2009). Hypertension was found to be present in 14.93% of the

diseased population as compared to 7.37% in the control population. This percentage is quite less as compared to other studies done but also correlates with various studies showing it to be the most comorbid condition prevailing along with RA (Innala et al., 2016). Physical inactivity is considered as a main cause for hypertension (Aziz and Yadav, 2016; Levytskyi et al., 2019). Diabetes was found in 3.98% of the disease population which is quite similar to that of a study done by Vij et al. (Vij et al., 2017) , wherein the prevalence was found to be around 4%.However there are studies showing a much higher prevalence of up to 13% in some studies (Tembe et al., 2008; Emamifar and Hansen, 2016; Singh et al., 2016; Levytskyi et al., 2019).

Failure to manage these co-morbidities effectively will have a serious impact on the RA patient. For example, the presence of co-morbidities may add delays to the care pathway, and the co-morbidities themselves may increase the patient's overall levels of disability, or even their risk of mortality. Moreover, Drugs like DMARDS,TNF i, CS, NSAIDS, are also found to increase the comorbidities thus prompting the judicious use of them by the Rheumatologists (Aziz and Yadav, 2016).

Risk factors: Furthermore, as it can be seen from the figure 9 that tobacco consumption was more prevalent in the males of RA population 72.45% which is supporting the work done by Vij AS. This could be considered as indicative for the causation of RA, at least among males. It has been well established that smoking triggers the genes that may cause an autoimmune disorder leading to RA (Plenge et al., 2007; Vij et al., 2017). There have been mixed reports on alcohol consumptions and causing of RA. In the current study, alcohol consumption was low in the diseased populations which is in support with reports suggesting a protective effect (Scott et al., 2013; Jin et al., 2014).

Moreover, it has been suggested that alcohol may been having protective effects in the development of other chronic disorder as well (Fekjaer, 2013). But there is a report as well showing to significant role of it in the development of RA. Here in this study as well no significant association has been found. There has been inverse association with alcohol consumption and cause of RA. But no significant difference has been found in the consumption of alcohol between both the populations (Karlson and Deane, 2012; Di Giuseppe et al., 2014a).

CONCLUSION

To conclude, this study confirms much higher frequency of comorbid conditions present alongside RA as compared to controls. In addition to Anaemia risk, among the most common comorbidities in RA were hypertension, Diabetes, thyroid dysfunction and a higher BMI. There are less studies focusing on the overall lifestyle and health of the RA patients. Future research should address the problem of decreasing coverage by rheumatologic care with an increasing number of comorbidities.

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Assessment of Plastic Degrading Ability of Microbes Isolated from Local Plastic Dumping Sites

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ABSTRACT

Polyethylene has become a serious environmental pollutant due to its highly non-biodegradability and has gained the attention of several researchers to find a way to degrade it. Here we have tried to degrade plastic using soil bacteria isolated from plastic dumping sites. A total of 20 bacterial strains were isolated from five different locations. Plastic degrading ability of all selected bacterial strains was checked on nutrient agar media containing 0.1% plastic powder. After primary screening, three bacterial strains P1, P5 and P11 were selected on the basis of growth behavior. Then these three strains were checked for their ability to degrade plastic. The biochemical and morphological characterization showed that the isolates with high degradation ability belong to genus *Staphylococcus*.

KEY WORDS: PLASTIC, BIODEGRADATION, SOIL BACTERIA, BIOCHEMICAL, *STAPHYLOCOCCUS*.

INTRODUCTION

Polyethylene is gaining much importance because of its properties like non-biodegradable, durable, light weight, low cost, strong and resistant to environmental changes (Gnanavel et al. 2012 Asmita 2015). They have many benefits as compared to other materials which make it suited for a variety of applications. But due to improper recycling and waste management system, it is creating environmental pollution (Hemashenpagam et al. 2013; Jayasiri et al. 2013; Gajendiran et al. 2016) and responsible for death of thousands of sea lives every year due to intake of plastic (Palmisano and Pettigrew 1992). Presently, we can get rid of plastic by incineration, dumping and recycling but all these methods fail to manage due to surplus demand in various sectors. So we have to focus on some ecofriendly methods. These methods include photo-oxidation, thermo-oxidative degradation and biodegradation. Out of these methods, biodegradation is considered as the cheap, environment friendly and most acceptable method for plastic management. After biodegradation, photo-oxidation can be considered as it utilizes UV radiations and microbes. Apart from these two methods, thermo-oxidation is not used commonly due to high requirement of energy (Yang et al. 2011).

During biodegradation, microbes get attached to microbial surface form colonies and start process of enzymatic degradation. Microbial enzymes break down the hydrolytic bond of polymer and convert it in monomer, dimer or trimer which is a simple form. Microbes again degrade it by aerobic and anaerobic metabolism and form carbon dioxide, water and methane (Tokiwa et al. 2009) as end product. Molecular weight of polymer plays an important role in its degradation as the physical properties

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of the polymer are determined by its molecular weight. With the increase in the molecular weight, degradability gets difficult. Some microbes like *Pseudomonas* can degrade styrene which is also responsible for triggering their metabolism (O' Connor et al. 1995).

Some other microorganisms like *Micrococcus luteus* and *Masoniella* were also identified for similar type of degradation (Sivasankari et al. 2014). So, to address such severe environmental problems, many researchers all over the globe are showing their interest in the optimization and production of biodegradable plastic (Jangra et al. 2018). Efforts towards conversion of plastic into usable forms like compost are also in progress (Albertsson et al. 1987). Many microbial species are studied by researchers which are able to degrade present polymer by using it as a substrate. It includes *Pseudomonas, Streptococcus*, fungus, *Micrococcus* and many other microbial species are known for their plastic degrading ability (Swift 1997). Therefore, researchers are trying to find out the ability of microbial enzymes for polymer degradation. The present investigation was conducted in the Department of Biotechnology, Government College, Hisar with a major goal to explore the natural properties of microorganisms isolated from plastic dumped soil for degradation of plastic.

MATERIAL AND METHODS

Soil samples and soil dumped plastic were collected in a test tube from different parts of Hisar (Haryana) and brought to laboratory and were preserved for further processing. Samples were collected from different garbage sites and dumped soil. Polyethylene bags were collected from local market and used after cutting into small pieces. Plastic powder was prepared in the laboratory and used in the media.

Isolation: In a conical flask, 99 ml of distilled water was taken. Then 1.0 gram of soil and plastic samples were transferred into it. The mixture was then serially diluted (Cappuccino and Sherman., 1996). Dilutions were made by adding 9 ml of sterile water to make 1:10 dilution, adding 1ml of the 1:10 dilution to 9ml of sterile water makes a 1:100 dilutions and so on.

Medium for testing biodegradation of plastics: The Bushnell and Haas agar (Bushnell et al., 1941) was used for testing the ability of microorganisms in degrading plastics. The media was prepared by adding 0.2 g of $MgSO_4$, 1.0 g of KH_2PO_4, 1.0 g of K_2HPO_4, 1.0 g of NH_4NO_3, 0.02 g of $CaCl_2$, 0.05 g of $FeCl_3$, and 20 g of agar into 1000 ml of distilled water. One-gram plastic powder (polythene/polypropylene) was added into the Bushnell and Haas agar medium to make a final concentration of 0.1% (w/v) with pH 7.0 $\pm$ 0.2. Media was then autoclaved and poured into petri plates for solidification.

Petri plate method for isolation of bacteria: For the isolation of bacteria, prepared dilutions were spread on the petri plates. Further the cut polythene strips were placed on the media and incubated at 37°C until the growth observed in the plates. After period of incubation the diameter of bacterial growth for each isolate was determined. Colonies were selected and purified and stored for further use.

Primary Screening of LDPE degrading bacteria in liquid media: Twenty- five ml of liquid media, was dispensed in 100 ml Erlenmeyer flasks and supplemented with 0.1% plastic powder as a substrate and sole source of carbon. Selected bacterial isolates were inoculated into the flask. All flasks were incubated in incubator at 30 °C for 7 days. The bacterial growth of isolates was determined at 600 nm by measuring the OD using U.V-visible spectrophotometer.

Determination of Weight Loss: Pre-weighed discs of 1-cm diameter prepared from polythene bags were aseptically transferred to the conical flask containing 50 ml of culture broth medium, inoculated with different bacterial species. Control was maintained with plastic discs in the microbe-free medium. Different flasks were maintained for each treatment and left in a shaker. After one month of shaking, the plastic discs were collected, washed thoroughly using distilled water, shade-dried and then weighed for final weight. From the data collected, weight loss of the plastics was calculated. Experiments were also done on agar plate using plastic discs.

Characterization and identification of microorganisms: Identification of the isolates was performed according to their morphological, cultural and biochemical characteristics by following Bergey's Mannual of Systematic Bacteriology (Kandler and Weiss, 1986). All the isolates were subjected to Gram staining and specific biochemical tests.

RESULTS AND DISCUSSION

The starting materials used for identification of polythene degrading bacteria were decomposed waste and polythene wrapped with soil (Singh et al. 2016; Rosario and Baburaj 2017; Jumaah et al. 2017). Result and inferences of experiments performed in this study are given below.

Isolation and identification of bacteria: From five diverse locations counting soil and decomposed waste, as many as 20 bacteria were isolated. Most of the bacterial strains were isolated from plastic dumped soil samples (Hisar, Haryana) and some from decomposed waste. The sample source, colony colour, and colony morphology are mentioned in Table 1.

Table 1. Morphological characterization of selected bacterial isolates

S. No.	Isolates	Sample source	Colony color	Colony morphology
1	P1	Plastic Dumped soil (Sample 1)	White	Round
2	P2	Plastic Dumped soil (Sample 1)	Orange	Shiny round
3	P3	Plastic Dumped soil (Sample 1)	Orange	Gummy spreading
4	P4	Plastic Dumped soil (Sample 1)	White	Round
5	P5	Plastic Dumped soil (Sample 1)	Cream	Round
6	P6	Plastic Dumped soil (Sample 2)	Cream	Round
7	P7	Plastic Dumped soil (Sample 2)	Light orange	Round
8	P8	Plastic Dumped soil (Sample 2)	Light orange	Irregular
9	P9	Plastic Dumped soil (Sample 2)	Yellow	Spreading
10	P10	Bare land	Yellow	Shiny round
11	P11	Bare land	Light orange	Round
12	P12	Bare land	Light orange	Round
13	P13	Bare land	White	Gummy spreading
14	P14	Govt. College	White	Gummy spreading
15	P15	Govt. College	Off white	Irregular
16	P16	Govt. College	Cream	Round
17	P17	Decomposed Waste	Orange	Round
18	P18	Decomposed Waste	Off white	Round
19	P19	Decomposed Waste	Orange	Irregular
20	P20	Decomposed Waste	Orange	Irregular

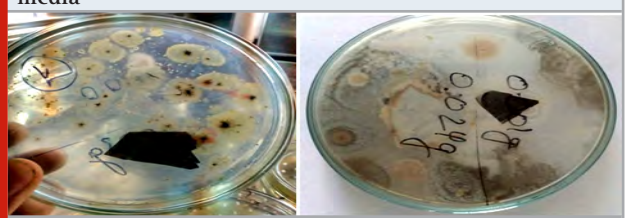
As most of the plastic degrading bacteria were collected from soil and plastic samples, it was concluded that soil and plastic waste are very good source for the isolation of plastic degrading bacteria.

Primary screening of plastic degrading bacteria: Solid media containing 0.1% plastic was used for the primary screening of selected bacterial isolates (Fig. 1). Degradation efficiency of selected isolates was recorded by measuring the growth diameter of bacterial colonies on growth media (Table 2) as described by Tokiwa et al. (2009) and Usha et al. (2011). Plastic degrading ability was considered to be high when diameter of bacterial colony was greater than 2.5mm, moderate when it was in between 1.0 to 2.5 and weak when it was below 1.0mm.

Table 2. Primary screening of plastic degrading bacterial isolates

S. No.	No. of bacterial isolates	Growth ability of bacterial isolates			
		Strong	Moderate	Weak	ND(no significant growth detected)
1.	3	++++			
2.	5		+++		
3.	5			++	
4.	7				-

Figure1: Growth of selected bacterial isolates on solid media



After primary screening, it was found that three bacterial strains showed good growth on MSM media. Five strains showed moderate growth and five strains grew very weakly. Seven strains did not show any growth on MSM media as depicted in Table 2.

Lab testing of plastic degrading capacity of microbes

Weight loss determination: A disc of 1cm was prepared using polythene bags and pre-weighed before inoculation.

Then, it is aseptically transferred in the liquid as well as solid media with selected strains and incubated as shown in Fig 2. It is considered that bacteria use plastic as their food material and hence responsible for degradation of plastic. It actually depends on the nature of bacteria, because their favorable environment varies according to different soil conditions. After one month of incubation, plastic discs were recovered from media, washed thoroughly using distilled water, shade-dried and then weighed for final weight. According to Katherison (2003), Pseudomonas species is able to degrade plastic by 8.16% and polythene by 20.54%.

Apart from it, some fungus like Aspergillus, have capacity to degrade plastic by 7.26%. Although, this is only the primary steps and these efforts are not sufficient to manage the environmental damage which is caused by the plastic but it can be further increased and improved with advanced technology. Aswale et al. (2010) showed 50% weight loss in polythene discs treated with *Phanerochaete chrysosporium* after 8 months of shaking at room temperature and pH 4. Microorganisms have secondary metabolism to transform or accumulate plastic like compounds. Microbes hydrolyze plastic by enzymatic methods, degrade the polymer to low molecular weight compounds and make them available for further assimilation (Shah et al. 2009). Some environmental factors like temperature and pH directly affect the action of enzymes for biodegradation.

Figure 2: Microbial degradation of plastic in liquid medium after 15 days of inoculation

From the data collected, weight loss of the plastics was recorded as shown in Table 4. After lab testing of these selected strains, it was found that P1, P5 and P11 have strong plastic degrading ability as compared to other strains. The average reduction in weight of the different plastic samples by various isolates used is the present investigation was 4.0 mg (Table 4).

As bacterial strains P1, P4, and P11 showed maximum plastic degradation, so these three strains were further selected for gram staining and biochemical analysis. The results of gram staining and other biochemical tests are depicted in Table 5.

Table 4. Reduction in the weights of the plastic samples after one-month incubation

S. No.	Isolates	Initial weight (g)	Final weight (g)	Wight loss (mg)
1	P1	0.0632	0.0619	13.00
2	P2	0.0459	0.0451	8.00
3	P3	0.0321	0.0321	0.00
4	P4	0.0721	0.0710	11.00
5	P5	0.0734	0.0734	0.00
6	P6	0.0132	0.0130	2.00
7	P7	0.0432	0.0430	2.00
8	P8	0.0546	0.0546	0.00
9	P9	0.0342	0.0339	3.00
10	P10	0.0312	0.0312	0.00
11	P11	0.0159	0.0148	11.00
12	P12	0.0239	0.0239	0.00
13	P13	0.0231	0.0230	1.00
14	P14	0.0612	0.0612	0.00
15	P15	0.0354	0.0350	4.00
16	P16	0.0564	0.0564	0.00
17	P17	0.0431	0.0430	1.00
18	P18	0.0514	0.0514	0.00
19	P19	0.0311	0.0310	1.00
20	P20	0.0438	0.0438	0.00
Mean	0.0424	0.0421	2.85	
SD	0.0175	0.0175	4.28	
SE	0.0039	0.0039	0.96	

Table 5. Biochemical analysis of the selected bacterial isolates

Tests	P1	P4	P11
Gram staining	Gram positive	Gram positive	Gram positive
Catalase	Positive	Positive	Positive
Gelatin	Negative	Negative	Negative
Mannitol	Negative	Negative	Negative
Urease	Negative	Negative	Negative
Citrate	Positive	Positive	Positive

All the three strains showed positive response towards gram staining, catalase and citrate test while these were found to be negative for gelatin, mannitol and urease test. Microbes are responsible for degradation of a lot of stuff in nature. They are also actively involved in polythene degradation in natural conditions, but microbes can be tailored in lab for improvement of their plastic eating capacity. This can be possible by using optimization strategy. Combination of different pH, temperature,

and media composition with different stains can make it happen. We have also made some efforts in this direction. During this study, we selected three strains of bacteria P1, P4 and P11 isolated from plastic dumped soil samples. These strains were capable of eating plastic. The plastic degradation ability of these bacterial strains can be further improved using biotechnological tools. The biochemical analysis showed that all the bacterial strain identified belong to the genus Staphylococcus as per Bergey's Manual.

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Possible Zoonotic links for Corona Virus Disease-19: An Updated Review

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ABSTRACT

COVID-19 has spread in over 210 nations and territories shortly after China. On 29 February, 2020, World Health Organization listed it in a category of high risk and on 11 March 2020 it was designated a pandemic after the declaration being Public Health International Emergency on 30 January, 2020. The countries around the globe are working and making efforts to control and contain this virus. The outbreak yet again proves the capabilities of the zoonotic contaminations. Although the substantial evidence advises possibility of preliminary emergence of zoonotic evolution, it is early to make confirmations on the role hosts that are intermediate such as turtles, pangolins, snakes and the other wild animals in origin of the SARS-CoV-2. Moreover, in addition to bats, the natural host of numerous coronaviruses like MERS-CoV and SARS- CoV. The facts gained from the previous episodes of SARS-CoV and MERS-CoV are being subjugated to retort the virus. Recognizing the probable emergence of zoonotic link and exact mechanism that is responsible for the initial spread will aid to design the control and preventive strategies against the spread of SARS-CoV-2. The present review explains about the SARS-CoV/ COVID-19 with particular focus on role of the animals, zoonotic and the veterinary links with the control and prevention strategies based on the approach of One Health.

KEY WORDS: COVID -19 , SARS –COV-2 , ANIMALS , TRANSMISSION , VETERINARY.

INTRODUCTION

Earlier in the days of December where the people were planning to welcome The New Year along with the Chinese New Year on January 2020 the media houses reported the suffering of many individuals with an intermittent and a clustered situation of pneumonia of which the origin was still unknown to the world in Wuhan, China. Subsequently, after recording the first case in the month of 12 December 2019, the causal agent was easily identified as one of those from the family of Coronaviridae moreover on 12 January 2020, World Health organization declared it to be a fast scattering virus as the 2019 novel corona virus and the Novel Coronaviral pneumonia and CoV- related diseases were addressed as "Covid 19" by the WHO on 11 February 2020 (Du et al. 2020; Gralinski and Menachery 2020).

Later this year the emerging virus was also addressed as the "SARS CoV-2" by the CSG or the "Coronavirus Study Group" of the International Committee as on Taxonomy of the Viruses. On 12 March 2020 the world health organization finally declared the condition as pandemic that is threatening to the mankind to a very far extent (Chatterjee et al. 2020; Zheng 2020; Phadke and Saunik 2020; Rundle et al. 2020). As today, "SARS CoV-2" is believed as seventh coronavirus which is infecting humans. While other coronaviruses include the following OC43, HKU1, 229E, NL63, SARS-CoV and the MERS-CoV. Among all those the MERS-COV and

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SARS-CoV are zoonotic yet have concluded in massive outbreak due to the very high mortality rate in past two decades whereas the others usually are associated with the illness of minor upper respiratory tract (Wei et al. 2020). Sometimes this leads to the complicated disease when stirring in the immunocompromised individual. (Villamil-Gomez et al. 2020).

To be specific the culinary habits of the Chinese people includes the consumption of various wild animals. The general motivation which is responsible for such consumption by humans in China is because of the belief that consumption of few wild animals and their driven products hold certain medicinal values an also promotes human health in many aspects (Harypursat and Chen 2020). The circumstantial proof which links the first ever case of novel coronavirus to Huanan a south seafood market that offers numerous exotic live animals moreover our former knowledge that the coronaviruses are derived from animals helped us to conclude that the possible zoonotic communication in the SARS-CoV-2. However, it is early to conclude anything as our knowledge of the main source of such infection is restricted (Jalava 2020).

To discover the origins of SARS-CoV-2 would aid us to loosen the actual mechanism that is responsible for the preliminary transmission. After the attainment of a remarkable progress in a developing field concerned with as well as a highly precise lab based diagnostics , much consideration that has been surfaced on emerging operative vaccine and therapeutics for obstructing person to person transmission, old age contagions and the healthcare care employees infection (Chen et al. 2020), which is serious for developing suitable preventive and the regulate strategies against fast dispersal of SARS-CoV-2 contamination. Looking at advantageous propositions of the hydroxychloroquine the multicentric randomised learning that is ongoing to assess its operative as prophylactic measure in limiting the secondary SARS- CoV-2 contaminations along with related clinical indications evolution plummeting general spread of virus (Mitja and Clotet 2020).

Initiating from central of china, SARS-CoV-2 pandemic did not only spread in the 369 cities of China and only crossed the international margins within a very short period from December to March 2020. On 2 May, 2020, COVID-2019 has triggered persons in almost 210 nations and major territories in Asia, Africa, North America, Europe and the Latin America (Rodriguez-Morales et al. 2020a; WHO 2020). Thus, because of a very high rate of transmissibility all over the world it was hence declared as an emergency of public health of international interest by the World Health Organization on 30 January, 2020 and soon after as a pandemic (DuToit 2020; Habibzadeh and Stoneman 2020; Liu et al. 2020a, 2020b; wood 2020; WHO 2020).

In the beginning of the 21st century the other coronaviruses like MERS-CoV and SARSCoV, in the year 2012 and 2002 respectively have been the reason for (SARD) severe acute respiratory distress outbreak however, the present Covid 19 affected the population in larger numbers leading to a number of around 4.71 million confirmed affected as well as death toll by 17 May, 2020 of 0.31 million (WHO). These Statistics are relatively higher than the cases of MERS-CoV A and SARS-CoV however with lower fatality rate. The current situation of the pandemic has significantly affected the economies around the globe moreover, intensively affecting the developing nations. This pandemic not only adversely affected the operations of the world like disappointed multinational businesses, interrupted global trading, transportation, trade, tourism but has resulted in reduction in the income derived from the market, (Ayittey et al. 2020).

China is known as a home for many farms which rears several species of animals like deer, porcupines, snakes, civets, turtles, foxes, minks, bamboo rats, bears and birds. These kinds of farms can be helpful in understanding and knowing the origins of the SARS-CoV-2 (Zhai et al. 2020). Before addressing dogs, snakes and pangolins as the host of the SARS-CoV-2, a bunch of principles known as Koch's assumptions have to be fulfilled. Thus, it is absolutely unethical to discard these animals without proper conclusive evidence of the SARS-CoV spread from animals to humans (Brownlie 2020). The current reports of the SARS-CoV-2 in the animals such as cats, dogs and one tiger have concluded in needless fear among general public along with pet owners moreover have affected the welfare of the animals, (Parry 2020). The current compilation in brief highlights bound SARS-CoV-2, causing increasing cases of Coronavirus (COVID-19) in humans related to the role of that of animals, zoonotic aspects, veterinary importance and noticeable prevention and strategies of control that focuses on the one-health tactics to refrain and fight the situation of virus.

The virus of SARS-COV-2: The coronaviruses are RNA viruses with positive sense. The newly developed SARS-CoV-2 is a member of order Nidovirales, the family Coronaviridae, the sub family of Orthocoronavirinae under this belong four genera known as Alphacoronavirus, Betacoronavirus, the Gammacoronavirus and lastly the Deltacoronavirus are characterised. SARS-CoV-2 have its place to genus Betacoronavirus and the subgenus of Sarbecovirus. The SARS-CoV-2 and the MERS-CoV also belongs to the genus of Betacoronavirus. However, the SARS-CoV-2 is quite different that the mentioned two on genetic levels. SARS-CoV-2 is said to be 88-89%

identical to the two-bat origin of SARS coronaviruses. (bat-SL-CoVZC45) and bat-SL- CoVZXC21, also known as ZC45 and ZXC21), while it is said to be 82% identical to the humans SARS-CoV Tor2 in addition to the human SARS-CoV BJ01 2003 at nucleotide level as (Drexler et al. 2014; Hu et al. 2017; Hu et al. 2018; Chan et al. 2020; Malik et al. 2020a).

Only around 50 to 51.8% identities were observed between the MERS-CoV-2 and SARS-CoV and 79% between the SARS-CoV and SARS-CoV-2. Furthermore, the molecular level of the phylogenetic analyses showcases that SARS-Cov-2 is closer to bat origin of SARS-CoV (Mohd et al. 2016; Ramadan and Shaib 2019; Ren et al. 2020; Malik et al 2020a). The highly advanced and in-depth study of the genome identifies the presence of 380 substitutions of amino acids between the arrangements of the SARS-CoV-2 the (HB01) in contrast to equivalent consensus arrangements of SARS-CoV and the SARS-CoV-2 viruses. The amino acid substitution may have made contributions to the functional along with the pathogenic deviation.

The range of Host: The coronaviruses do not only infect humans but also animals of both wild and domestic kind the infections most of the times remain sub-clinical (Ji et al. 2020a; Li et al. 2020a; Salata et al. 2020). However, this clinical form differs from colitis in horses, cattle and swine, the upper breathing tract disease mainly in cattle, felines, dogs and poultry in addition to common cold to a highly fatal infections of respiration in man (Dhama et al. 2020a, 2020b). Midst the main four genera in coroaviridae family, the alphacoronavirus and the betacoronavirus commonly contaminate mammals and possess probable origins of bat meanwhile the Gammacoronavirus and the Deltacoronavirus contaminate fishes, birds and mammals and the assumption is that they are from pig origin (Woo et al. 2012; Hu et al. 2017; Cui et al. 2019). The Betacoronavirus genes have probable zoonotic pathogens like the SARS- CoV and the MERS-CoV that possess bats as the primary host moreover secondary hosts such as civet cat for the former and dromedary camels for latter (Wang and Eaton 2007; ArGouilh et al. 2018; Ramadan and Shaib 2019).

Many CoVs that has been improved or recovered from the birds like Bulbul coronavirus HKU11, Wigeon coronavirus HKU20, Night- Heron coronavirus HKU19, Munia coronavirus HKU13 and the general moorhen coronavirus HKU21. The general swine contaminating infections from coronavirus includes Transmissible Gastroenteritis Virus, Porcine Coronavirus HKU15, Porcine Epidemic Diarrhoea virus and the Porcine Hemagglutinating Encephalomyelitis virus that are being testified across globe (Ma et al., 2008). Furthermore, another list of species of animal are also states harbouring CoVs like horses, swine, cattle, cats, dogs, rabbits, rodents, camels, ferrets, birds, mink, snake (like krait and Chinese cobra), bats, frogs, hedgehogs (the Erinaceus europaeus), marmots, Javan or Malayan or Sunda pangolin (the manisjavanica), while many othe significant wild animals and the role they play as reservoir must be given even more attention by the world (WHO 2203; Dhama et al. 2014a, 2014b 2020a; Monchatre- Leroy et al. 2017; Ji et al. 2020a; Malik et al., 2020b; Xu 2020).

SARS-CoV-2/ Covid19: zoonotic links, veterinary, animals and transmission

Animals being affected by Coronavirus: As it is known that the coronaviruses possess a wide animal hosts, few species of animals also harbour the pathogens and a very few of such animals get affected by serious contamination (Cui et al. 2019; Andersen et al. 2020). The coronaviruses such as rat hepatitis virus, guinea pig virus, rat sialodacryoadenitis coronavirus and the rabbit coronavirus are few important viruses that are responsible for the enteritis, hepatitis and the breathing infections in the lab animals. Amongst a large number of animals, the bovine coronaviruses (BoCoVs) possess zoonotic potential known as being different from the asymptomatic children and found affecting few domestic animals and the wild ruminants where bloody diarrhoea among adult cattle, calf diarrhoea in the neonates and the respiratory form of fever in all groups irrespective of age in cattle are global implication (Zhang et al. 1994; Suzuku et al. 2020).

The Feline coronavirus affects respiratory tract, the major nervous system, gastrointestinal tract, abdominal cavity in order to produce infectious peritonitis and enteritis (Tekes and Thiel 2016). The Canine enteric CoVs of the Alphacoronavirus and the Betacoronavirus genera bother enteric and the respiratory region respectively, (Erles and Brownlie 2008; Licitra et all. 2014).In poultry industry the virus of infectious bronchitis, the member of the Gammacoronavirus genes results in extensive loss in economic operations by developing respiratory issues, infection in the urinary tract and the reproductive issues (Dhama et al. 2014a, 2014b). The swine acute diarrhoea syndrome CoVs a member of the Alphacoronavirus genes that produces very serious enteritis in the suckling piglets that causes significant mortality.

Upon such genomic study, the virus was found in 95-96% of identical to the horseshoe origin of bat (Rhinolophus sp.) CoVs and the known HKU2 coronavirus (Wanh and Jin 2020). Furthermore, it has been suggested that there is a possibility of the host skipping from bats to the pigs by crossing species obstacle either by recombination of genes or by developing changes at level of (RBD) receptor binding domain (Zhous et al. 2018; Yang et

al. 2019).Among all the species of animals another new coronavirus named as the SW1 is recognised by making use of the technology of panviral microarray in liver tissues of captive beluga whale (Mihindukulasuriya et al. 2008).

Zoonotic links and Animals of SARS-CoV-2: Coronaviruses in he past has crossed the barrier of species during MERS and SARS outbreaks, and therefore SARS-CoV-2 appears to be an outcome of the barrier of the species jumping for third time. Among the CoVs, the current zoonotic ones like MERS-CoV, SARS-CoV and the SARS-CoV-2 has gained higher significance due to the seriousness of the disease in the humans moreover their global blowout (Rothan and Byrareddy 2020). The development of the new CoVs and the extensive host range it possesses is may be because of the instability of replicase enzyme, the RNA is dependent on the RNA polymerase, the polybasic furin cleavage site and the O-linked glycans, deficiency mechanism of proofreading, the advanced mutations rate in RBD of the genetic recombination and the spike gene (Su et al. 2016; Chen 2020; Patel and Jernigan 2020). Multiple Research show that the SARS-CoV and the SARS-CoV-2 both make use of the ACE2 as a common cell access receptor (Zhou et al. 2020a). Because of the mutation in the region of the RBD of gene S of CoVs, the range of host gets prolonged in order to infect the other host species whether humans or animals, the transmissibility and pathogenicity of the virus may get increased on altered in the future becoming a situation of global concern. (Chen 2020; Patel and Jernigan 2020).

While conducting a search of the origination of the SARS-CoV-2, the observation was made that initially the infected person has a general exposure spot. The Wet seafood market of wholesale in south china in the city of Wuhan is known for restaurants that offers large and small domestic and wild animals moreover live animals that includes rabbits, bats, poultry, snakes, turtles, pangolins,hedgehogs, marmots and badgers for consumption by humans. (Hu et al. 2015; Hui et al. 2020; Ji et al. 2020b; Liu et al. 2020a, 2020b; Lu et al. 2020b; Wang et al. 2020; Wu et al. 2020b). The inferences in the beginning from Wuhan's seafood marketplace hypothesised the source of animals' attachment and the wild animals for spill over of the SARS-CoV-2. The results of the Research indicated the chances of zoonotic basis because the CoVs circulate between certain species of animals, vertebrate and the humans as there are wide range of hosts. There was an assumption that the SARS-CoV-2 was initially transmitted from the animals to humans, and maintaining a human to human spread (Hui et al. 2020; Ji et al. 2020a; Nishiura et al. 2020).

In case of the MERS-CoV, there are evidences which states biological RNA is released by the nasal secretions and the faeces also from the milk, stating the danger of food borne spread of the MERS-CoV (Reusken et al. 2014). Moreover, a number of camels that are offered for slaughtering in few studies showcased evidences of shedding of nasal MERS-CoV (Farag et al. 2015). Furthermore, the chances of SARS-CoV-2 as being a food borne infection of CoVthat is spread by route of respiration (Jalava 2020). The literature documents showed that there are a few of origin of bat SARS-CoV and were likely enough to infect human beings. As noted formerly, bats were overserved to be transmitting the SARS-CoV and the MERS-CoV, hence the researchers predicted the role played by bats in the transmission and origin for the present pandemic of the SARS-CoV-2 (Fan et al. 2020a; Malik et al. 2020a; Wong et al. 2019; Zhou et al. 2020a).

Presently it is understandable that SARS-CoV-2 is intimately related to coronavirus of the bat which is believed to be a preservation of the hosts of the former CoVs related to SARS. Therefore, SARS-CoV-2 may have been developing from the recombination of sequential combinations that occurs between the viruses related to SARS and precursors. On the basis of the usage of the codon bias snake was projected as the reservoir of the SARS-CoV-2 (Ji et al. 2020a). Nevertheless, this projection was contradicted by few researchers. This was the cause for questioning the presence of any intermediate host (animal) which can be responsible for the spill over of zoonotic transmission among humans (Weiss and Leibowitz 2011; Murdoch and French 2020).

In a similar way not just only from bats the coronavirus is associated with SARS that was transmitted from the humans to swine (Chen et al. 2005). It is relevant to state that the swine had been one of the predominant species for evolution in the past of various strains of Influenza A when existing in an intimate association with the avian and the species pf human and as the CoVs of bats that infect pigs, possibility of development of any novel virus involving corona and influence can't be left out including the present situation of the increasing SARS-CoV-2 cases, explorative studies and researches are required for such hypothesis (Brown 2001; Dhama et al. 2012; Malik et al. 2020a).

The given conditions at any time, swine that serve as the vessel of mixing of viruses carrying influenza (Ma et al. 2009) needs to be considered with safety as it remains in the proximity with human and the cycles of domestic sylvatic that involves contact with various wild animals and the circumstances might get worse (Ma et al. 2008). Nevertheless, for the current time, researches of Shi et al. (2020) have not disclosed any important vulnerability of swine to SARS-CoV-2. Civets, camels and bats have been in the list of current animal carriers of the human infections of CoV (Cui et al. 2019). In the latest, the bats

(Wu e al. 2020b) and the pangolins (Zhang et al. 2020a) are believed to be the possible origin source of the SARS-CoV-2 (Andersen et al. 2020).

Till now the real intermediate nature and host that led to emergence are yet to be discovered and explored. The two scenarios of the development of SARS-CoV-2 are taken into projections. In the first the natural selection of the viruses which might have been occurring in any animal host formerly before the spread in the humans and secondly it is the natural selection of the viruses that has occurred in the humans after zoonotic spread (Anderson et al. 2020). The advanced researches that involves the cuture of cell or the animal models may help in answering these hypothesis (Ge et al. 2013; Anderson et al. 2020).

The Bats: Bats are considered to be the superlative reservoir hosts for the CoVs, as the virus are continuously present in bats and are asymptomatic. They tend to travel across the jungles in quest of and spread virus to different kinds of hosts as they get in contact with (Fan et al. 2019). In china bats are of significant purposes as they not only are sold for consumption purposes but also for medicinal purposes and the wild bats are often used to attaincompounds derived from bats. Even though bats possessmedicinal value, a severe risk is posed by them for acquiring novel zoonotic infection (Riccucci 2012; Wassenaar and Zou 2020). In present situation novel coronavirus pandemic, findings of the laboratory confirmed that the SARS-CoV-2 is constituted 96% alike to bats Cov at genomic therefore bats might be the principal source of the zoonotic spill over (Tang et l. 2006; Rodreiguez Morales et al. 2020b; Zhou et al 2020a).

The Pangolins: The bats are not the only reservoir of coronavirus but Malayan pangolins are also isolated for coronavirus and RBD is the protein S of the SARS-CoV-2 was very similar to the of the Pangolin-V thus, the pangolins may be the intermediate host of the novel coronavirus (Xiao et al. 2020). The conclusions of Zhang et al. (2020b) also support the stated research. Most interestingly, coronavirus secluded from the pangolins (SRR101068377 and SRR10168378) didn't possess RRAR motif. SARS-CoV-2 virus secluded from the individuals that are infected showcased a huge alikeness to Beta COV/Yunnan/ CoV/RaTG13/2013 virus that is compared to those that were secluded from pangolins (Li et al. 2020b). Such researches suggested that pangolins had little probability of acting as one of the intermediate hosts of the SARS-CoV-2. Further the studies required to recognise intermediate host and in order to confirm their part in the development and origin of the virus in man.

Felines and Canines: Until now the SARS-CoV-2 infection is identified in two dogs, where both are reported from the city of Hong Kong (Almendros 2020a). The primary case was identified and reported in a Pomeranian dog which was 17-year-old which gave a positive RTPCR outcomes in both the cases of nasal and oral samples (Almendros 2020a; American Veterinary Medical Association 2020). Although the serological test done initially gave a negative result, the blood tests that were carried out later in the future gave a weak positive outcome. The reason for this might be because of the fact that the development in the antibody may take 14 days or more (Almendros and Gascoigne 2020b).

A research report of the seroconversion in dogs showcased that animal has formed antibodies against the SARS-CoV-2. This suggests that the virus imposed weak infection in dog and such result was due to the immune system. Therefore, the studies suggest an unbiased and true contamination in the case of dogs which was transferred from humans to animals (Almendros and Gascoigne 2020b). In a similar way one such case of SARS-CoV-2 contamination was identified in German Shepherd in Hong Kong. Interestingly, that both mentioned cases of the canine SARS-CoV-2 contaminations were identified with positive owners of SARS-CoV-2 (American Veterinary Medical Association 2020). Presently, there are no substantial proof that the dogs get infected from SARS-CoV-2 or are able to spread the virus to the humans (Almendros 2020a).

In another case where animals were again involved was of two cats, one from Hong Kong and the other from Belgium was tested positive for the virus of COVID 19 (American Veterinary Medical Association 2020). Scientist from the Harbin Veterinary Research Institute has stated that the cats can be infected with the SARS-CoV-2 under the conditions of experiment and are able to transmit to the other cats that are susceptible and are pet together in the same house,(Mallapaty 2020; Shi et al. 2020). The research was on the basis on the experimental inoculations and might not stand accurate in the natural conditions. None of those cats who were infected displayed any symptoms or signs or any sickness, that indicates their low capability for spreading the infections (Mallapaty 2020).

The serological study that was carried out among the cats from the city of Wuhan noticed the attendance of the neutralizing antibodies of SARS-CoV-2. This study specifies that the cats are not immune to the infection of SARS-CoV-2 under the natural conditions that results in a response that is antibody (Zhang et al. b). Nevertheless, among cats that were tested positive, an advanced titre of the neutralizing antibodies was seen in cats who were staying in a close contact with the owners who were

SARS-CoV-2 positive (Zhang et al. 2020b). In a recent case, Malayan tiger from the Bronx Zoo in the New York city was also identified as a SARS-CoV-2 positive. This big cat is also assumed to be contaminated with the virus because of the positive tested asymptomatic keeper of the same zoo. The carnivores were being tested for the virus when they began to display of minor respiratory sickness (United States Department of Agriculture 2020).

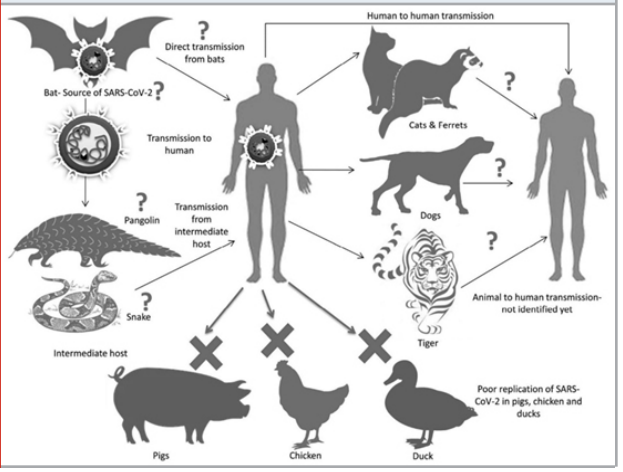
Animal and human interactions are a risk factor: Many researchers stated the traditional way of cooking in China as one of the reasons responsible in a way for the situation of infection of the novel coronavirus in human beings because as stated by the customs of Chinese food that the live slaughtering animals are believed to be even more nutritious however the same time, many get in contact with the any or all types of pathogens that are possibly present (SARS-CoV, Rotavirus, Nipah virus, Highly Pathogenic influenza virus) that could be present in the food items that are offered (FAO/WHO 2008). Human and animals' interactions on a regular basis either in market place or in animal industry without taking better ecological biosecurity were believed as an important risk factor for the increasing number of zoonotic diseases, predominantly in the communities that lives in the rural china in the southern part (Daszak 2020).

After such reports, china has temporarily banned the sale of wild animals and trading of bats after the breakout of novel coronavirus. In addition, Wuhan's food market has been closed to refrain the spread virus and development of novel variant of any virus can be barred. Furthermore, it has been advised to ignore any such contact with wildlife and farm without use of protective personal gear (Benvenuto et al. 2020). In addition to this, the need that arises is to waft strategies for surveillance and guidelines for prevention to get an in-depth analysis of the betacoronavirus. Specifically, in the bat family of Rhinolophus as in previous cases of MERS, SARS and currently SARS-CoV-2 pandemic that has created panic (Daszak et al. 2020). Therefore, the bat appears as the source od origin or natural reservoir of the SARS-CoV-2 (Li et al. 2020) which leads to the zoonotic infection in people through a middle host that is yet to be decoded with current investigations on the ferrets, pangolins and probably snakes. Nevertheless, the future developments might disclose the original intermediate hosts of the virus SARS-CoV-2 that is responsible for the zoonotic spread (Almenndros 2020a; Dhama et al. 2020d; She et al. 2020; Zhang et al. 2020a, 2020b).

Animal Models: Even though the current studies on animal models lack for the SARS-CoV-2, a current study explored the utility of the rhesus macaque, non-human primate as a model that carries the SARS-CoV-2 studies. Earlier these non- human primates helped in calculating the antivirals and the vaccines against the MERS-CoV (de Wit et. Al 2020). The work process on the SARS-CoV-2, rhesus macaques showcased the settlement of the infection of SARS-CoV-2 which possessed high amount of virus in the rectal swabs and oral-naso.

Apparently, the lesions of the disease in radiographs of the lungs and the clinical signals lastingup to a period of 16 days evidently proved the effectiveness of diseases and helping in establishing and testing the antivirals and vaccines (Munster et al. 2020).The isolation SARS-CoV-2 from the dogs is also noted (OIE. 2020). Currently, Shi et al. (2020) have stated the susceptibility of cats, dogs, ferrets and various domestic animals to SARS-CoV-2 by an experiment inoculation and stated that SARS-CoV-2 is poorly replicated in pigs, chickens, ducks and dogs however efficiently in cats and ferrets. Cats can transmit the infection via their droplets (Shi et al. 2020). Nevertheless, exact exploration needs specific models, specifically animals with the ACE2 receptors which are similar to those people (Andersen et al. 2020).

Figure 1: Zoonotic links of SARS-CoV-2. Bat has been reported as the reservoir source of SARS-CoV-2. The intermediate host is not yet ecucidated cleary and presently snake and/ or pangolins are reported to the intermidate host. Reports regarding the transmission of SARS-CoV-2 from human to animal have been speculated. Study also shows that SARS-CoV-2 replicate poorly in pig, chicken and duck while ferrets and cats are susceptible



Establishing an accurate animal models will aid in understanding process of the disease and also developing therapeutics and prophylactics (TBRI 2020; Dhama et al. 2020c). The non-human primates are believed to be suitable models of the human diseases, where in case of exploration to-pathogenesis of disease and the immune response, other modes are preferable (TBRI 2020). The non-human mice, hamster and primates (Gretebeck and Subbarao 2015) were used as the animal models for the MERS and SARS, and few may have probability

in SARS-CoV-2. The golden Syrian hamster has been investigated for studies on protection of the vaccine against the strains of SARS-CoV-2 (Roberts et al. 2008). Moreover, advised to be the capable animal model that reveals the pathogenesis and CoV pathology along with the efficiency of the vaccine that is to be tested. In case of transgenic animals like mice have better relativity of stimulating the SARS-CoV-2 as there are presence of structural differences in the ACE 2 receptors in some species of animals to which the receptors are binding the domain of protein of spike of SARS-CoV-2 (Liu et al. 2020b; Wang 2020).

On modelling the receptors of ACE2 between some animals like cats, orangutans, ferrets, pigs, monkeys, species of bats and human possess alike levels of affinity for the virus SARS-CoV-2 that is based on the similarity of structures of the receptors of ACE2 (Jarvis 2020). Therefore, these might have probable role that can be used as the animal's models with furthermore investigative researches. The smalls animal's model is preferable generally like rabbits and mice as it is cheaper and easier to manipulate and easily available (Dhama et al. 2020c). Primarily, mice were thought to be challenging because of the distinction in the ACE2 receptor usage pattern, however for SARS-CoV-2 the transgenic mice now are believed to be the applicable models (Li et al. 2020a, 2020b Wang 2020; Zhou et al. 2020b).

Animals like Ace2 knokout mouse, inbred mouse, Tmprss2 knockout mouse, Transgenic HLA mouse and Stat1 knockout mouse are being speculated as he animal modesl for the COVID-19/ SARS-CoV-2 (Hoffmann et al. 2020; Taconic Biosciences 2020; Wang 2020).

Control and Prevention: In the past and the current situations of Ebola, Zika, viruses of Bird Flu, Nipah (Munjal et al. 2017; Dhama et al. 2012, 2018; Singh et al. 2019), and knowledge earned from past threats of the coronaviruses (MERS- and SARS-CoVs) along with the advance in field of science have paved the path to counter developing pathogens like SARS-CoV-2. For this situation, high efforts are made to control and contain the transmission of the virus that is haunting the lives of many around the globe and posing a situation of pandemic even now. Therefore, works for strict vigilance, rapid diagnosis, quarantine procedures and appropriate isolation are being done to refrain the further transmission. Moreover, enhanced surveillance and observation, intensive care unit, better medical facilities, rapid communication, networking programs, awareness programs for the public, efforts to establish effective anti-viral, drugs and vaccines are being speculated and discovered optimally.

Global collaborative readiness and efforts to handle the further emergency to a level of the situation of pandemic capability along with the proper and applicable heath approach to fight this virus are being used effectively (Bonilla- Aldana et al. 2020, Dhama et al. 2013a; 2013b; 2020a; 2020c; Malik et al. 2020a, 2020b; Rodriguez-Morales et al. 2020c). The vaccines look long lasting solution for the pandemic. Nevertheless, presently there are no such vaccines available to fight against it (Shang et al. 2020; Chen et al. 2020). Ideas are being motivated from the structures of virus, pathogenesis and the connected coronavirus (Ahmed et al. 2020; Shang et al. 2020).

There are various vaccines that are being evaluated by the different companies and institutes (Shang et al. 2020) with few of them under trials. Moderna, Ma, Cambridge, USA a biopharma company initiated the mRNA-1273 vaccine collaborating with the CanSino in Hong Kong the Special Administrative Region, situated in China (Flanagan 2020). Birmingham' s University of Alabama (UAB), USA, AI, Birmingham in coordination with the Altimmune Inc., Md, USA, Gaithersburg, is working on development of an intranasal antiviral against the COVID-19 and termed it AdCOVID on pattern of the pandemic vaccine for influenza and inhalation anthrax (Hansen et al. 2020). Clover Biopharmaceuticals firm, China, Chengdu has come up with SARS-CoV-2 protein that is based on the subunit vaccine (Clover Biopharmaceuticals 2020).

As on 20th March, 2020, The WHO has calculated over 44 candidates for vaccine that targets SARS-CoV-2 among these only a few are under the clinical observation while others are under development by institutions and companies.Theyincluded live attenuated, formaldehyde inactivated, protein subunit, DNA, m-RNA, VLP, replicating, and non-replicating vector-based SARS-CoV-2 vaccines. Adenovirus type 5 vector vaccine by CanSino biological Inc., Hong Kong Special Administrative Region, China, and Beijing Institute of Biotechnology, Beijing, China, andLNP-encapsulated mRNA vaccine developed by Moderna/NlAlD, Bethesda, Md, USA, is under phase-I clinical evaluation.

Few in pre-clinical stage of clinical evaluation against COVID-19 include DNA plasmid vaccine by ZydusCadila, Ahmedabad, India, DNA plasmid vaccine through electroporation device by Inovio pharmaceuticals, Plymouth Meeting, Pa, USA,DNA vaccine by Takis/Applied DNA Sciences/Evvivax, Rome, Italy, formaldehyde inactivated alum vaccine by Sinovac, Beijing, China, live attenuated virus vaccine by Codagenix, Farmingdale, New York, USA/ Serum Institute of India, Pune, India, MVA encoded VLP by GeoVax/BravoVax, Smyrna, Ga, USA, Ad 26 by Janssen pharmaceutical companies, Beerse, Belgium, ChAdOx1 by university of Oxford, Oxford, UK, adenovirus-based NasoVAX expressing SARS-CoV-2 spike protein by

Altimmune, Gaithersburg, Md, USA, Ad5 S (named as GREVAXTM) by Greffex, Aurora, Co, USA, oral vaccine by Vaxart, South San Francisco, Car USA.

Animal derived Drosophila S2 insect cell expression system using VLPs as protein subunit vaccine by ExpreS2ion, Horsholm, Denmark, S protein based by WRAIR/ USAMRIID, Fort Detrick, Md, USA, another S protein by AJ Vaccines, København, Denmark, S-trimer by Clover Biopharmaceuticals Inc., Chengdu, China/ GSK, Brentford, UK, peptide based vaccine by Vaxil Bio, Toronto, Ontario, Canada, S protein through baculovirus production system by Sanofi Pasteur company, Swiftwater, Pa, USA, full length S trimers nanoparticle with Matrix M by Novavax, Rockville, Md, USA, gp-96 backbone based vaccine by Heat Biologics, Morrisville, NC, USA, or University of Miami, Miami, Fl, USA, Sl or RBD protein based by Baylor College of Medicine, Houston, TX, USA, adjuvanted microsphere peptide vaccine candidate by University of Saskatchewan, Saskatoon, Saskatchewan, Canada, LNP encapsulated mRNA encoding RBD by Fudan University/Shanghai JiaoTong University/RNACure Biopharma, Shanghai, China, sa-RNA (small activating ds-RNA) based COVID-19 vaccine by Imperial College London, London, UK, among others (https://www.who.int/blueprint/priority-diseases/key-action/novelcoronavirus-landscape-ncov.pdf?ua=l).

Though till April 1st, 2020 US Food and Drug Administration (FAO) had not announced any confirmed commercial therapeutic or prophylactic vaccine against SARSCoV-2, nevertheless they have enlisted few potential vaccine candidates which are currently under either preclinical or clinical trials such as mRNA-1273 by Moderna Inc., Bethesda, Md, USA, Inovio's DNA vaccine INO-4800 against COVID-19 by Inovio Pharmaceuticals, Plymouth Meeting, Pa, USA, along with Ology Bioservices, Alachua, Fl, USA, BNT162 a mRNA vaccine by BioNTech, Mainz, Germany, plantbased COVID-19 vaccine by Medicagor Quebec, Quebec, Canada, oral recombinant COVID-19 vaccine by Vaxart, South San Francisco, Ca, USA, li-Key peptide COVID-19 vaccine by Generex Biotechnology, Toronto, Ontario, Canada, among others (PrecisionVaccination2020). (https://www.precisionvaccinations.com/vaccines/coronavirus-vaccines).

Till the time vaccines are set, alternate diseases control and prevention strategies require the focus. There is a requirement for strengthening he capacity and infrastructure that is build with trained employees, health workers and services to recognise the patients that are affected by SARS with isolation of the patients after they are suspected of the COVID-19. In order to apply any measures for prevention the primary step is check the case with speed and accuracy. While the confirmation of the deadly case, the guidelines of the Centre for Disease of and Prevention or CDCmust be implemented. As suspected cases are good source of any nosocomial blowout, the health employees should follow the precautionary practices when handling COVID-19 cases. It is notable that a facility with the adverse air pressure is advised for keeping the confirmed cases of SARS-CoV-2.

Applications of the telemedicine that have supervision and, monitoring, tele-visits, consultation and interpretation (Serper and Volk 2018) is proven to be effective in modifying the lingering diseases. Tele-model is being applied to the current situation of COVID-19 specifically in the isolated area that have limited medical reach that saves both the resources and manpower (Au 2020). Even though the infections of respiratory tract and transmission of virus from the naso-oral secretions is well explained, the primary isolation of the SARS-CoV in China of a positive patient displayed the importance of digestive tract in the conjunction to respiratory tract. Ever since, when planning the strategies for control, this alternative path of transmission of virus was kept in mind that includes a focus on asymptomatic patients and clinical sufferers or persona who have mild or no signs (Gu et al. 2020).

CONCLUSION

The markets for live animals, just like one in the South China, Huanan sea food market, will remain to be the ideal point which encourages the inter-species interaction between the domestic and wild animals. Therefore, the probability inter-species spread of the CoVcontaminations at hot spot is a centre of worry to the human being because of the adaptive recombination of genetic, that is present in these viruses. The perpetual ban on trade of wild animal should not be executed as it would only move the trade to black market. It is better to control and monitor the trading of wild animals rather than a complete ban. The spread of the newer zoonotic contaminations like the SARS-CoV-2 is unavoidable in the days to come.

Thus, the international and local regulatory bodies must develop and enforce mechanisms for diseases' control that is effective enough to decrease the probability of human acquaintance to the wild animals. The breakout of the SARS-CoV-2 is another example which proves the presence of any intimate but upfront interaction between the animals, people and environmental health which can potentially result in development of live threatening pandemic. The recent years have showcased the destructive capabilities of few zoonotic coronavirus contaminations like MERS, SARS, and presently the

SARS-CoV-2 which needs call for the applications of the framework of One Health to safeguard the humankind from the evolving pathogens soon.

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The Hard Times of COVID 19 and Previous Major Health Emergencies for Speculating a Post COVID India

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ABSTRACT

Human existence reminds of the hard times that every nation of earth has witnessed in the series of hostilities such as world wars, great depression, cold war etc. But in 2020 it is not wars that have led to halting the lives and mobility of mass in the era of globalization; an invisible enemy becomes the root cause of this. COVID 19 became the paradox of today's emergency. The situation reminds mankind of early preparedness and speculation of the invisible threat beforehand which needs an attention over improving daily behavioral practices, health care facilities and insurances, economic alternatives and boosting of science and technology in the field of research and medicine. The following article looks on to the literature of previous health disasters and what we should learn in combating such kind of emergencies in the future. The study becomes an important discourse for drawing inferences from previous major health emergencies the world has witnessed and in third world societies like India how the negativity of a tragic phase can be tackled as a responsive behavior. Case history of previous infectious diseases and literature reviews becomes the methodology for the manifestations of proliferating the measures to combat such instances in a developing country like India. The article would work on the measures for growing consciousness and alertness among the masses for generating unique behavioral advances which would demonstrate the importance of health education in times of crisis. Various insights such as online education, working from home, adequate importance for research and scientific temperament, behavioural changes in terms of social distancing, frequently washing hand, change and greeting habits, rejuvenation of ecology and environment becomes a positive factor for a new normal in today's tragic times.

KEY WORDS: COVID 19, EMERGENCY, INDIA, PARADOX, RESEARCH.

INTRODUCTION

Canadian president's statement of the corona virus's hard times being the new normal depicts a path that shall be forsaken onto indulgence of a positive insight on digging up the word normal. The new normal seems to have horrifying effects of which the imagination does not mentor thinking of the present reality. If reality sustains as it is, the very structure of mankind would be changed shortly without a possible vaccine being developed in the upcoming days. The disease COVID 19 originated in Wuhan, China of which pneumonia started among the mass whose cause could not be determined initially. This was eventually reported to the WHO by the Chinese on 31 December 2019 (Jiang et al, 2020).

Subsequently it gained recognition of a possible emergency on 30 Jan 2020, when it was declared a Public health emergency. The name of the disease as COVID 19 was announced on 11 February 2020 (ibid). From then onwards the disease has been spreading all across the globe like wildfire, killing hundreds and thousands of people. The specter of the disease has created a situation of the pandemic which frightens the existence of mankind

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after an accumulation of events such as world wars, great depression, etc. Human indulgence in certain areas of life emancipates tragedies of latent functions such as the paradox of GM crops relating to contract farming, the use of science and technology in terms of generating weapons of mass destruction (Specter, 2009; Jiang et al, 2020).

The Novel Corona Virus tends to hit the respiratory system hard that makes people difficult to breathe. The disease tends to spread through droplets of human beings and the governments and the World Health Organization is concerned for safeguarding the humans having provided guidelines from safeguarding oneself against the virus. The disease being unable to surpass by medicine has sustained for many months to date and this act of longevity creates an act of challenge for science to come with an antidote or medicine. The disease has brought scientists and researchers into a single unit for its eradication. This worldwide pandemic creates certain dialectical reasoning of behavior among the mass. These dialectics of the disease can be seen on the societal ground too for precaution and mobility. This involves frequently washing hands, maintaining social distance, wearing masks, isolating in house and being less mobile. These various social practices have created room for awareness among the mass, which would lead to profound impacts in the future (Prosser et al, 2020).

METHODOLOGY

The following communication focuses on the existing literatures of health emergencies and draws a perspective on how to secure ourselves from threat such as diseases and looks onto the social structure that will be juxtaposed in a post COVID 19 India. This becomes a necessity study because the world has seen many such crisis and for the betterment of our future it becomes an important principle to learn from health tragedies such as COVID 19. Third world countries like India, where population becomes an important factor, health emergencies can be an act for rampant dissolution of the society with huge population and therefore various risks generated throughout should be taken into consideration and which would bring effective precautionary knowledge for tackling such kinds of crisis in near future. The use of case history in terms of previous health emergency is taken and with this the underpinnings of the societal behavioural insights are seen which lets us to understand Indian society in terms of health crisis. The emerging ongoing trends are taken from India during the lockdown to focus on the parameters of health, education, scientific temperament and economy.

Major infectious Diseases of the 20th and 21st century:
HIV: The occurrence of HIV was found in early 1920 in a region to be now called the Democratic Republic of Congo. (Gao et al), 1999 in their study has found that the chimpanzees had the source of HIV-1 and this virus had at certain conjuncture has crossed species from chimpanzees to humans. In terms of human cases related to HIV, it was classified as the first verified case in 1959, when a man's blood sample from the Democratic Republic of Congo was analyzed and termed to be the detection of HIV (Science, 1998). The disease as deadly as it is has created havoc across the world but to date no such medicine is discovered for its cure. But medicines have come in the limelight for delaying the probability of the virus. COVID 19 on the other hand is also a novel zoonotic disease that is caused by a corona virus from China in the year 2019 (Mackenzie et al, 2020). This virus has certain similarities and dissimilarities as compared to HIV. Both the virus can target the T lymphocyte cells (Wang et al, 2020). But this does not denote they are the same. Corona virus has a significantly lower spontaneous mutation rate than HIV (Wang et al, 2020).

HIV poses a serious ontological question to Sars Cov-2 for the probability of discovering a potential Vaccine and reminds us how unsafe we are if we look into the world through a futuristic lens. HIV has created a certain amount of consensus in the world that awareness about the disease in today's time seems to be more effective. Knowing that the disease spreads through blood and other bodily fluids, measures are to be taken in terms of blood donations where safe and hygienic sexual intercourse became habitual practice aftermath the spread of the disease. This reflexes the awareness that has been injected into the population to be able to halt the proliferation of the disease. Therefore on this account the corona virus implies a similar situation, where social distancing and washing hands should become a behavioral insight for the people across the globe, and this should be carried out through awareness. The dystopia of living in a world with viruses and diseases as a new normal must be something that people should be aware of and start reasoning for their wellbeing from this (COVID et al., 2020).

H1N1: The H1N1 influenza virus, S-OIV appeared in 2009 in April in Mexico (Gibbs et al 2009). The swine flu was also a zoonotic disease as compared to COVID 19. The disease had the symptom of fever, chills, cough, and headache which are also the symptoms of COVID 19. The disease tends to spread through coughing and sneezing. However anxiety and fear were created among the mass because of unavailability of Vaccine in the beginning, but later at the end of 2009, a vaccine was developed which would protect in the future flue seasons. Covid 19 in terms of H1N1 flu there was no immunity among the mass at the starting of the outbreak, and this led to a crisis of how fatalities in 2,84,000 death all across the globe were recorded (Dawood etal, 2012). The starting of H1N1 gave people a new platform in the form of the internet to know the doubts on the disease. The population started using the internet for logging into various online platforms for discussing issues relating to the disease. The outbreak of H1N1 saw demand for health and hygiene and this was generated through the sales of sanitizers, orange juice, thermometers, vitamins, cough and cold remedies, tea, soup (Todd Hale et al, 2009; Wang et al., 2020).

Ebola: Ebola had its origin in the year 1976 in Nzara Sudan and Yambuku, Democratic Republic of Congo (Mazid et al, 2016). These are the two regions where the disease has parallel origination. The virus had its largest repercussions during 2014, which was the optimum since 1976. The outbreak started in West Africa, which had the largest of all facilities including deaths. The virus had enormous socio-economic impacts on the society, where the GDP was hit very hard, impacting the trade and commerce in the continent especially Sierra Leone, Liberia, Nigeria, and Senegal. The tough times of African society in the nuances of Ebola justifies the fact that health is one of the important sectors, which if developed would stimulate the growth of other economic sectors. To restrict the outbreak from spreading to other parts of the world, the movement of people was restricted.

The disease had worked in a latent function for encompassing integration and solidarity among the African nations to fight against the disease with unity through deploying military and humanitarian missions by the member states. To restrain from the disease unambiguous consensus has been built among the mass as the days passed on. Washing hands frequently became a habitual practice that persisted in the human consciousness aftermath of the disease, (Contezen et al, 2015). In places like Haiti and Ethiopia, washing hands became a matter of educating and socializing the children. (Gamma et al, 2017; Wang et al., 2020).

Lessons learned from previous health disasters: The various health disasters and pandemics such as Spanish Flu, Ebola, and H1N1 etc. are zoonotic diseases and humans getting more proximity with wild might lead to situations of viruses travelling from the wild. And to this from the various health disasters we see that human history and natural history is encompassed within the same realm of existence. And this is to say that the rampant anthropocentric effects on the environment and wild may lead to devastating outcomes for the human health and wellbeing.

Threats such as these speak of a global collaboration. These are some things that can't be halted in the borders within the nation, but these are something that tends to spread. And therefore there is requirement of the whole global community coming together to combat in such situation and help each other through necessary requirements, living aside the political and ideological affiliations and differences. Another important dimension that health disasters such as this show that scientific temperament is very important for every nation. And scientific research should be boosted through investment in research and development to fight against an invisible enemy of virus (WHO, 2004; Wang et al., 2020).

Health crisis such as these shows that the more we adapt towards behavioral changes for staying away from the virus the more positive results this portrays. In terms of Ebola we have seen the more the people followed the measures of social distancing and new habits of greetings such as subtle bow or rub of the palms to the more flamboyant gestures of bumping bottoms or throwing your hands in the air in a star shape, or the gentle foot-pat half-greeting, half-dance, rubbing left foot to your counterpart's right have seen to be a positive gesture in stopping the spread of the disease (Prosser et al, 2020).

India– on a post COVID 19 world: The COVID 19 as deadly as it is not the first pandemic to have affected the world. In India the plague outbreak of 1898 and the Spanish flu of 1918 illustrated how disease and policies around it are never just medical but are embedded within the politics of socioeconomic fabric of the economic and social order we live in. Both the crisis brought out a sharp critique against the then British colonial government and its negligence of the medical structure of the country. At great the human costs of the pandemic provided a momentum to rebuild solidarity to reevaluate alliances. Living from how the state responded during the crisis, crafted new dimensions in the critique of colonialism and imperialism. This moment of crisis is the neoliberal unending consumption-led world that might once again lead us to question the tenants of economic organizations of such capitalist growth with minimal attention to basics necessity parameters of health and education (Kickbusch, 2001; Prosser et al, 2020).

The COVID 19 saga started in India by late January 2020. In the initial phase, the Indian Council for Medical Science Research on 15 February 2020 started the sentinel surveillance for COVID 19 and on March 11, 2020 the process of universal screening started and the travelers who came to India would be in the purview of 14 days quarantine. On 17th March 2020, closure of all educational institutions, theaters, museums took place. Eventually lockdown was implemented all across the nation to stop the proliferation of the disease. This subsequently led to drastic changes in the habitual life of the people. Everything including all social and economic activities came to halt in a brink of a second. The lockdown resulted in an adverse effect on the economy which led to submerge growth.

The sectors which affected India and will continue to affect in the future are the tourism sector, aviation sector, manufacturing sector. But the COVID 19 saga could boost certain sectors like the digital and creative sector encompassing a diverse range of activities from telecommunications to advertising and computer programming to broadcasting. Economically drastic alterations would occur as demand subsumes all over the world and India too. And hence production process comes to pause with low production across the nation. The falling rates of crude oil can be beneficial for the government in procuring crude at a lesser price and stocking in the reserves. The government on the other hand must look into it and provide aids and loans for sustaining the SMEs. People would now consider the risk of traveling and in the upcoming days they would emancipate entrepreneurship and farming in their local places. Local trade and commerce for local consumers through local markets could be boosting the market economy (Kanitkar, 2020).

The current time and space have created a paradigm for hyper usage online social networking sites and online media sites such as Netflix, Amazon Prime and this hypothesizes a situation of movies of regional and Bollywood getting released in these online platforms rather than theaters in the purview of COVID 19. Again the situations of learning and teaching through internet access can become an opportunity for alternative measurements in terms of education in which the open and distance learning is best known from years as such type of learning and teaching mechanism could be considered as a safe working strategy for managing mass aspirations for higher education without necessarily affecting large congregation (Menon, 2006; Prosser et al, 2020).

The process of lockdown has also boosted localism within the country, where family time is seen to be given much importance by the population. The school-going children are to be provided special care amidst their family by their parents and the parents would also get to know their children effectively and would understand their problems and trauma because of being too intimate in the hours of lockdown and will result in necessary care and nourishment. A shift in the pattern of mobility is seen during the starting of 2020, where there was an increase in globalization of trade and commerce and movement of people across the globe (ibid). But in the aftermath of the disease this pattern has changed into negligence mobility of the mass and confining themselves in their respective residences and moving in and around only for essentials. This scenario has led to the betterment of the environment and ecosystem where the wild is rejuvenating implying that nature is healing.

The carbon immersions dipping can lead to a better scenario for the cities such as New Delhi, Kanpur etc. The real issue that the nation faces during the times of pandemic is the issue of unemployment. The unemployment crisis would however uproot many of the migrants to lose their jobs in the cities and move to their respective native places. But this can also benefit the rural economy if the workforce ventures into the small and medium enterprises in their respective villages. The present situation puts the demographic dividend into a state of jeopardy, where the working-age population instead of working is into lockdown mode. Behavioral practices such as washing hands frequently, covering faces with masks could become a regular practice and may become part of a normal life. Greeting without shaking hands with Namaste may become a regular practice (Singh et al, 2020).

The COVID 19 saga in India now sets back importance for the health sector, in which research and development, as well as treatment and medical resource, becomes important in certain tough times. Thus this pandemic not only challenges the global health system but our commitment to equality and human dignity. The poor must be looked upon by the government because they are the ones most affected and to tackle this there should be the implementation of universal basic income. The consensus of compulsory health insurance becomes important in certain tough times which the government must be keen upon in the future. But this can also be turned to an asset by learning new things and upgrading skills through the internet and other digital modes. People might get themselves involved in reading, writing, poetry, music, dance, yoga, farming eventually leading them to generate new ideas, and thus these ideas could be used in terms of generating income for sustenance. During a crisis like this, India needs to invest in education and most certainly community education and community participation. This can be carried out through media, guiding people towards betterment (Khongsai etal, 2020).

The paradigm of pandemic determines the element of risk perpetuated in India. The risks have greater repercussions in terms of gathering food, medicines, and has confined to the lockdown itself the poor and weaker section suffers. The affluent classes are the ones which possess the potentiality to tackle the risk or get rid of the risks, whereas the weaker sections of the society including pregnant women, children, old aged population, and physically disabled ones are seen to have risks within themselves to mitigate the upcoming risks (Marshall, et al 2020). The phenomenon of risk becomes asynchronous to society. Modernization has introduced such risks in the society where proximity with humans towards animals has led to an imbalance in the ecosystem and catastrophes in the form of risks (Beck, 1992).

Modernization and globalization as a product of Science and Technology become the forefront in times of pandemics as such. Thus when the probability of finding a vaccine in a certain period becomes slim to none questions the manifestations of scientific temperament across nations (Alsop et al, 2020). Thus the iron cage rationality phenomena portrayed by sociologist Max Weber prevails in the discourse of modernization in the eyes of Beck known as reflexive modernity, where the very fundamental functions of modernity come at stake, where unintended and unforeseen side effects of modern life backfire on modernity (Wimmer et al, 2006). Development without investing much on research might lead to latent consequences. Therefore the need of the hour demands stimulation of scientific research. A new normal can be built upon what we have discovered under lockdown, about making a living and living well (Prosser et al, 2020).

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Covid19 and Public Health Disruption: Living in the New Normal

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ABSTRACT

The COVID – 19 pandemic has unleashed unprecedented afflictions worldwide. The disease spread has led to a dramatic loss of human life and crumbled public health systems, global supply chains and economies. Consequently, the global perspective is undergoing drastic alterations, birthing a comprehensive demarcation between pre- and post-COVID-19 era. The changed face of the global community will be characterized by tightly wound travel, exchange and venture boundaries; and more sustainable and resilient approaches to facilitate pandemic preparedness and better health systems. This article highlights how the virus has staggered the public health aspect of human life. With its far-reaching repercussions having overwhelmed the health care systems due to increased demand and grave under-preparedness, the pandemic has also disrupted the global economy causing millions of enterprises to face an existential threat. Simultaneously, school closures and lockdowns have crippled the education system, and the majority of service sector has been rendered unfunctional due to travel restrictions, border closures and confinement measures. These changes have disrupted the livelihood patterns of populations and increased the susceptibility of countries already dealing with humanitarian crises or emergencies towards the ill-effects of the disease. This article is an outcome of a careful review of literature which focuses on how the pandemic has impacted public health, hindered health care delivery and imposed new behavioral and business mandates upon us in the future. As the tragic scenario unfolds, it has become imperative for agencies and stakeholders worldwide to come forward and take initiatives to restore the health systems and formulate well-thought-out and inclusive strategies.

KEY WORDS: COVID -19, PANDEMIC, QUARANTINE, SOCIAL DISTANCING, HEALTH CARE SYSTEMS, PUBLIC HEALTH, SUSTAINABILITY.

INTRODUCTION

The malady which stemmed from China's Wuhan in late 2019 has continued well into 2020 with no indications of ending anytime soon. With over 36 million cases of the infection and more than one million deaths, the disease has morphed into a global crisis. Governments worldwide have formulated and executed quarantine measures along with other interventions, such as lockdowns, to suppress the transmission. According to the data provided by the Indian government, levying an early lockdown prevented 1.4 to 2.9 million infections and around 37,000 to 78,000 deaths. (The Economic Times, 2020).

Such drastic measures, although necessary, gravely impacted all social orders and elements of practicality. During a pandemic, like COVID-19, it is common that there is a substantial level of panic buying, which increases the demand of certain items such as toilet papers, kitchen towels, and canned food. (El-Terk, 2020) There was an apparent change in buying behaviour in

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the FMCG market, which laid bare the shortcomings of global supply chains. Pertaining to quarantine measures, a major concern was the delivery of essential items in severely affected zones. (Singh et al, 2020) Along with disruptions in the economic landscape and livelihoods, the catastrophe has even disrupted essential health services delivery leading to adverse public health outcomes.

This article is based on a meticulous review of literature aiming to deduce the impact of the pandemic on the public health landscape, the system and process of health care service delivery and also on the business and behavioral practices which seem probable to be imposed upon us in the future, as put forth by many experts. Adopting precautionary regulations such as social isolation, increasing sanitation, and employing strict quarantine measures have proved to be beneficial in containing the virus. (Khachfe et al 2020) Implementation of these interventions have become a major part of our day-to-day lives, and consequently many ponder that which of these pandemic-induced modifications will become a part of the post-COVID era.

Repercussions for Public Health and Associated Sectors: With no regard for out-patient services and hike in self-treatment or self-medication, the struggling health care providing facilities and poor health status of the people are a testament to the aftermath of the pandemic being more precarious than the outbreak itself. There have been changes in the health seeking behavior of the people, especially in case of major treatments such as surgeries, scheduled therapies, chronic treatments and others. The fear of the virus has also given rise to non-institutional deliveries, which further poses the burden of increased maternal and infant morbidity and mortality.

It was estimated that this loss of service will likely result in an additional 38 million unintended pregnancies, 679,864 child births, 1.45 million abortions (including 834,042 unsafe abortions), and 1,743 maternal deaths. (Mohitar, 2020) An expansion of vagrancy and in the frequency of different ailments is likely to be observed, attributed to the breakdown in immunization administration programs for polio, measles, cholera, human papillomavirus (HPV), yellow fever and meningitis, affecting at least 13.5 million people in 13 of the world's least-developed countries. (COVID-19: Massive impact on lower income countries threatens more disease outbreaks, 2020)

For assessing the impact of coronavirus on Chinese population conclusively summarized that the virus has mild to moderate impact on the mental health and quality of life among residents. (Zhang and Ma 2020). However, this study needs to be conducted on the wider section of the society before reaching a conclusive answer. An upsurge of over 21% was seen in crimes against women during the COVID-19 lockdown in India. Pertaining to unfit living conditions, many people have been subjected to unsurmountable mental strain and pressure – women due to increased household responsibilities, children due to changes in their lifestyle, young people due to job insecurity and loneliness, the elderly because of being a high-risk group and loneliness, migrant workers due to loss of livelihoods, frontline health care workers due to fear of contracting the virus and unbearable job pressures, and sexual minorities due to exclusion from government's policies and changes in their lifestyle. With more than 50% of its population under the age of 25 years, the country reports around 28 suicides each day, as per the data from the National Crime Records Bureau. Nearly 197 million Indians are known to suffer from some sort of mental disorder and COVID-19 has adversely affected the mental health of these individuals. (Sagar et al, 2020).

The virus has also invariably altered the definition of normally accepted social behavior standards. Most of us prefer shaking hands or hugging our acquaintances to greet them, but in the health emergency that COVID-19 has caused, it may be considered as an act of ignorance. (Colive 2020) In the wake of 'social distancing' measures to prevent person-to-person transmission, infected people are being advised to self-isolate or quarantine, which is especially difficult for people with extraverted or outgoing personalities. Being cut off from society can frustrate them and contribute to social incompetency, leading to a hike in the cases of mental and emotional episodes ranging from anxiety disorders to various forms of depression. Being virtually connected through telephone, video chat, or social media has become a coping mechanism. Telemedicine mental health visits, group visits, and delivery of care via technology platforms will be important components of stepped care for both acute crisis management and more routine communication and support. (Galea et al 2020).

The impact of the virus, disease-spread and its prevention measures has been drastic and multi-faceted. Individuals and corporations alike are participating in quarantine practices and evolving safer work structures and workforce redistribution, and compromising their operational activities to curb transmission rates and protect and safeguard the health of the public. However, COVID-19 disruptions do not affect all businesses equally. Some are deemed essential and remained open, while others were forcibly shut down. (https://www.hbs.edu/faculty/Publication%20Files/20-102_1c8a5b54-d400-4a8d-b136-d6890cf876dd.pdf) Essential services and businesses like clinical stores, centers, and emergency clinics remained open to support and strengthen the public health framework in the battle against this ensuing virus novelty.

Other activities vital to the civilization such as education, travelling, social engagements and businesses have also faced the brunt of the repercussions that COVID-19 entails. It has emerged as the biggest disruption in the education sector in history, making over 1.6 billion students staying out of school each day and demolishing opportunities, more so for the vulnerable groups, making them more prone to domestic abuse, lack of nutrition and mental strain. United Nations latest reports that closures of schools and other learning spaces have impacted 94 per cent of the world's student population, up to 99 per cent in low and lower-middle-income countries. (United Nations, 2020) Young and adolescent children have been confronted with immense strain with regards to their well-being and security pertaining to school closures,

changes in lifestyle and increased prevalence of nourishment uncertainty. With most economies expecting a contraction in their GDP till FY-2021, and negative growth rates reported for the first quarter of FY-2020, various speculations state that the efforts to curb hunger and poverty worldwide will be gravely pushed back. The World Bank estimates that 40 to 60 million more people will be living in extreme poverty in coming months, depending on the scale of the economic shock Due to evident changes in worldwide markets and consequently, lesser flow of money into the economy, the chances of an economic revival seem bleak for the short-term. (Pangetsu, 2020) This indicates that the public health systems of all countries are bound to be impacted to some extent attributed to shrinking economies and negative GDPs and vice versa.

As the health care workers continue to fall sick, people continue to succumb to the virus and economies continue to crash, it has become crucial for institutions and individuals around the world to come forward and present a united front to mitigate this 'external shock' to the global health care system. Researchers, scientists and health care professionals from the world over have joined hands to seek treatments, or develop vaccines, or curate mechanisms for early detection of the disease. Companies and businesses have come forward to aid the frontline healthcare workers, keep the supply chains moving and providing financial and/or material aids to the vulnerable groups. Various communities, faiths, religions and people from all walks of life, have done and are doing in helping curb the spread of this virus novelty. As the COVID-19 keeps endangering the global community, various innovative initiatives have been put forth with multi-faceted collaborations between diverse stakeholders.

Some of these initiatives include WHO Solidarity Call to Action, UN Call for Technology Solutions, Coalition for Epidemic Preparedness Innovations Urgent Call for Vaccine Preparation. WHO Global Research and Innovation Forum towards a research roadmap, Global COVID-Zero Initiative, followed by tons of other undertakings. These efforts range from fund-raising for COVID-response to finding a vaccine to contemplating digital health or technology-based solutions to tackle the virus. World Economic Outlook Update states that the global community must act now to avoid a repeat of this catastrophe by building global stockpiles of essential supplies and protective equipment, funding research and supporting public health systems, and putting in place effective modalities for delivering relief to the neediest. (World Economic Outlook Update, 2020)

CONCLUSION

Drawing on recorded evidence from the past outbreaks and the current scenario as it unfolds, analysts contend that the far-reaching effects of COVID-19 will leave a profound and seemingly irrevocable impact on the global community. As the health care systems worldwide stand on the verge of or have undergone a collapse, reinforcement of robust and pro-active public health measures emphasizing on healthcare promotion and prevention is crucial. These mechanisms will act as pillars upholding the trust of the population looking forward to experiencing normalcy. Surfacing, spreading and even re-surfacing of COVID-19 have made the world realize the value of building a resilient public health care system, that can withstand any crisis or external challenges.

The repercussions of not acting now, will give rise to a challenging 'Domino' effect, not only on the health care delivery process, but also on public distribution system, economy and education. Multilateral foundations such as the WHO, UNICEF, UNESCO, and many more serve as role models in evaluating and implementing appropriate strategies. The current arrangements have necessitated the formulation of migration laws, emergency preparedness, risk assessment and better monitoring and evaluation mechanisms in our existing health systems, processes for which are underway in many nations. The disease has travelled from one small region to around 213 countries and territories and is bound to resurface again, as evident by second waves in China, France, Singapore, Norway, Spain and regions of Australia.

For the short term, the focus should be on saving lives and livelihoods by devising appropriate and sustainable containment strategies, followed by phased reopening and resumption of normal life. For the long term, the region should opt for a GDP-centric growth rebound by resetting and reprioritizing its healthcare policies. The economies should collaborate and move towards a more prepared, more sustainable and more resilient healthcare and business landscape, which would facilitate the attainment of desired growth while focusing on capacity building.

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Analysis of Pathogenic nsSNPs in Human SNCA Gene Associated with Parkinson Disease

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ABSTRACT

Parkinson's disease (PD) is second most common motoric neurodegenerative disorder, which affects population above age 65. It is characterized by the loss of dopaminergic neurons from substantia and the presence of intracellular. SNCA is a protein encoded by 5 exons with total transcript length of 3041 bps maps on 4q21.3-q22. SNCA gene is considered to be involved in regulation of dopamine release and transport, induces fibrillization of microtubule associated protein tau, and exert neuroprotective phenotype in non-dopaminergic neurons by inhibiting both p53 expression and transactivation of proapoptotic genes leading to decreased caspase-3 activation. Polymorphisms in Alpha-synuclein (SNCA) gene have been associated with Parkinson disease. In this study, computational analysis of pathogenic SNPs of SNCA gene has been performed to identify and analyze the deletrious SNPs using bioinformatics approach. We obtained pathogenic SNPs data from dbSNP database. We employed consensus tools SIFT, PROVEAN, Condel, PolyPhen-2 to predict deleterious pathogenic nonsynonymous SNPs, Pathogenic mutants A30P (rs104893878) and G51D (rs431905511) shows deleterious by all four tools and three tools respectively. These predicted pathogenic deleterious nsSNPs are expected to have impending functional influence and may contribute in understanding the functional roles of SNCA gene associated with Parkinson disease.

KEY WORDS: NSSNP, ALPHA-SYNUCLEIN, SNCA, IN SILICO ANALYSIS.

INTRODUCTION

Parkinson's disease (PD) is second most common motoric neurodegenerative disorder (Mhyre TR et al, 2012; Bisaglia, et al, 2010) which affects 1–2% of the population above age 65 and 4–5% above age 85 (Bisaglia et al,2009).It is characterized by the loss of dopaminergic neurons from substantia and the presence of intracellular (Vilar et al,2008). SNCA is a 14.5 kDa, 140 a.a protein encoded by 5 exons with total transcript length of 3041 bps maps on 4q21.3-q22. The other members of synuclein family are SNCB and SNCG mapped to human chromosome 5q35 and 10q23.2-q23.3 respectively (George,2002).

Architecture of SNCA protein reveals the presence of N-terminal region composed of incomplete KXKEGV motifs, extremely hydrophobic NAC domain and highly acidic C-terminal domain8. At physiological conditions, SNCA is believed to be intrinsically disordered monomer or helically folded tetramer10. Oligomeric structure of SNCA is considered as a toxic form but recent observation abolished this hypothesis, (Dettmer et al,2015). Hypotheses exist about toxic structural form of SNCA, but none of them are completely consensual. However, neurotoxic form of SNCA aggregates within neuron and spreads

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across the anatomically interconnected regions of PD brain through interneuronal transmission using various mechanisms (Recasens et al,2014).

Although SNCA is expressed predominately in brain, it is also expressed in heart, skeletal muscle and pancreas (Lücking& Brice,2000).Molecular function of SNCA is quite ambiguous. Based on its structure, physical properties and several hypotheses for the normal function of SNCA have been proposed. It is considered to be involved in regulation of dopamine release and transport, induces fibrillization of microtubule associated protein tau, and exert neuroprotective phenotype in non-dopaminergic neurons by inhibiting both p53 expression and transactivation of proapoptotic genes leading to decreased caspase-3 activation (Huang, 2018). However, missense mutations (especially A53T) in (Tang et al,2008). SNCA abolished the neuroprotective effect of SNCA and promote apoptosis by reversing the expression of p53 (da Costa et al,2000).

Due to the significant role of SNCA in FPD and other neurodegenerative disorders, premeditated to decipher the molecular evolution of SNCA, which infers the phylogenetic history of synuclein family with the help of its putative orthologs and paralogs. Analysis revealed the sarcopterygian specific origin of SNCA which suggested its lineage specific functional role. On account of this interest, a comparative sequence and structural analysis was performed to estimate the selection and functional constraints on SNCA. Evolutionary rate difference was coupled with structural information to infer potential functional changes and the impact of lineage specific substitutions. In addition, variations in domain topologies were explored by comparative analysis of known functional domains of SNCA protein. In light of the findings, it was hypothesized that the region from 32 to 58 of N-terminal lipid binding domain is the most "critical region" of SNCA from evolutionary, functional and disease pathogenesis perspective. Multiplications of the SNCA gene locus, including duplications and triplications, have been shown to cause autosomal dominant PD in which gene dosage determines severity and latency (Ferese, 2015).

This suggests that 1) the pathologic properties of α-synuclein are not dependent on mutations that alter the α-synuclein protein product, 2) overexpression of wild-type α-synuclein is sufficient to cause disease in a dose-dependent manner and 3) overabundance of α-synuclein may be a common feature of PD wherein smaller increases in α-synuclein, due to increased expression or decreased clearance, may contribute to sporadic disease (Singleton & McCormack,2007). So Alpha-synuclein (SNCA) is considered as the major causative gene involved in the early onset of familial Parkinson's disease (FPD) characterized by missense mutations reported A30P (;E46K, H50Q, G51D and A53T. SNCA is also deemed to be involved in various other neurodegenerative disorders i.e. Alzheimer's disease (AD), Lewy bodies' disease (LBD) and Muscular System Atrophy (MSA) (Kruger,1998, Hashimoto & Masliah,1999 Siddiqui, 2016).

MATERIAL AND METHODS

Datasets: The SNPs of the Alpha-synuclein (SNCA) gene were retrieved from the dbSNP database (Sherry ST, 2001). Keyword "Human SNCA" used as our search term. It is filtered by selecting variation class as SNV, function class as missense, clinical significance as pathogenic. Protein sequence of SNCA (uniport id: P37840) was retrieved from uniport database.

Prediction of deleterious and damaging nsSNPs: In order to predict the damaging or deleterious nsSNPs, multiple consensus tools were employed by using online tool VEP (http://www.ensembl.org/Tools/VEP). VEP advantages include, it can predict thousands of SNPs from multiple tools including SIFT, PROVEAN, Condel, and PolyPhen-2, at a time. Pathogenic nsSNP rs-ids were uploaded to VEP tool to get the prediction results.

SIFT: The algorithm predicted that the tolerant and intolerant coding base substitution based upon properties of amino acids and homology of sequence (Choi Y, 2015). The tool considered that vital positions in the protein sequence have been conserved throughout evolution and therefore substitutions at conserved alignment position is expected to be less tolerated and affect protein function than those at diverse positions., SIFT predicted substituted amino acid as damaging at default threshold score ≥0.05, while score < 0.05 is predicted as tolerated.

PolyPhen-2: This tool is predicting the structural and functional consequences of a particular amino acid substitution in human protein (Adzhubei, 2010). Prediction of PolyPhen-2 is based on a number of features including information of structural and sequence comparison. The PolyPhen-2 score varies between 0.0 (benign) to 10.0 (damaging). The PolyPhen-2 prediction output categorizes the SNPs into three basic categories, benign (score < 0.2), possibly damaging, (score between 0.2 and0.96), or probably damaging (score >0.96).

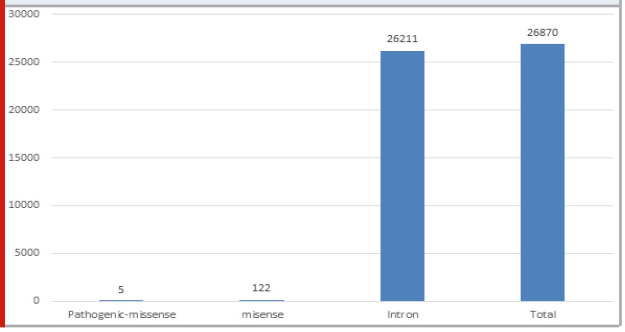
PROVEAN: This tool (http://provean.jcvi.org/) uses an alignment-based scoring method for predicting the functional consequences of single and multiple amino acid substitutions, and in-frame deletions and insertions (Choi Y, 2015). The tool has a default threshold score, i.e. -2.5, below which a protein variant is predicted as deleterious, and above that threshold, a protein variant is neutral.

CONDEL (CONsensus DELeteriousness): This tool evaluates the probability of missense single nucleotide variants (SNVs) deleterious. it computes a weighted average of the scores of SIFT, PolyPhen2, MutationAssessor and FatHMM (Hecht M, 2015).

RESULTS AND DISCUSSION

Five SNP-ids of pathogenic nsSNPs mapped in human SNCA gene was downloaded from dbSNP database of NCBI (Table 1), after filtering variation class SNV, function class missense and clinical significance as pathogenic, there were 122 SNP mapped to missense, 26211 SNPs mapped to intron, while 26870 mapped to total SNPs of different variation class (Figure 1). Some rsIDs are associated with multiple SNPs and therefore fall in different classes.

Figure 1: Number of SNPs in different function class of SNCA gene of human from dbSNP database



Predicting deleterious and damaging pathogenic nsSNPs: In order to predict the damaging or deleterious pathogenic nsSNPs multiple consensus tools were employed. Initially, online tool VEP was used (McLaren W, 2016). VEP advantages include: it uses latest human genome assembly GRCh38.p10, and can predict thousands of SNPs from multiple tools including SIFT, Condel, and PolyPhen-2, at a time. 5 nsSNP accession numbers were uploaded to VEP tool and the prediction results were taken on default scores of consensus tools based on sequence and structure homology methods: (a) SIFT (score <-0.5) (b) Polyphen (score >0.96) (c) PROVEAN (score< 2.5) and Condel (score >0.522). In order to get a very high confident nsSNPs impacting structure and function of SNCA gene, 2 nsSNPs (Table 1) are found to be deleterious or damaging by three prediction tools and 1 nsSNP were found to be deleterious by all four tools. These two nsSNPs rs104893878 of mutation A30P and rs431905511 of mutation G51D were further analysed by Netsurf tools for Protein Surface Accessibility and Secondary Structure.

Studies show a strong evidence about variants are found in SNCA gene involving with Parkinson disease (Jackson, 2003, Stijnen, 2016, Stijnen, 2016). This gene involved in the early onset of familial Parkinson's disease (FPD) characterized by missense mutations reported A30P, E46K, H50Q, G51D and A53T ((Appel, 2013, Kiely, 2015).

Table 1. Prediction of five pathogenic missense SNPs of SNCA gene using prediction tools such as SIFT, Condel, Polyphen and PROVEAN.

SNP-ids	AA-Change	SIFT (score)	PolyPhen (score)	PROVEAN (score)	Condel (score)
rs104893878	A30P	Deleterious (0)	Probably damaging (0.996)	Deleterious (-4.536)	Deleterious (0.906)
rs104893875	E46K	Deleterious (0.03)	Benign (0.003)	Deleterious (-3.477)	Neutral (0.364)
rs201106962	H50Q	Tolerated (1)	Benign (0.014)	Neutral (0.895)	Neutral (0.001)
rs431905511	G51D	Deleterious (0.03)	probably damaging (0.999)	Neutral (-2.302)	Deleterious (0.855)
rs104893877	A53T	Tolerated (1)	Benign (0.01)	Neutral (1.828)	Neutral (0.001)

Our investigation shows two pathogenic mutants very high confident nsSNPs impacting structure and function of SNCA gene, nsSNPs rs104893878 of mutation A30P found to be deleterious or damaging by three prediction tools and nsSNP rs431905511 of mutation G51D found to be deleterious by all four tools. These two nsSNPs may offer valuable information in selecting SNPs that are expected to have impending functional influence and pathogenicity.

CONCLUSION

Our investigation shows two pathogenic mutants very high confident nsSNPs impacting structure and function

of SNCA gene, nsSNPs rs104893878 of mutation A30P found to be deleterious or damaging by three prediction tools and nsSNP rs431905511 of mutation G51D found to be deleterious by all four tools. These two nsSNPs may offer valuable information in selecting SNPs that are expected to have impending functional influence and pathogenicity also eventually may contribute in understanding the functional roles of SNCA gene associated with Parkinson disease.

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Association Between Oral Contraceptive use and Some Biochemical Changes in Saudi Women

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ABSTRACT

Millions-of females worldwide use oral contraceptive pills (OCs) to improve their health outcomes and for family planning. Oral contraceptive (OCs) use has been associated with an unfavorable impact on lipid metabolism by increasing the level of atherogenic lipids, which are strongly associated with cardiovascular disorders. The aim of this study was to evaluate the effect of oral contraceptive pills on lipid profile and liver function in Saudi females. A total of 55 Saudi women aged (20-40)years old were divided into two groups: users oforal contraceptive pills (OCs, N=30) for at leastone year and non-users (NOCs, N=25). The current study was based on the questionnaire having the data includes age, height, weight, body mass index (BMI), blood pressure, physical activity, duration of OCs, and types of OCs. Blood specimen from both groups were drawn after 8 hours of fasting to estimate serum glucose, lipid profile (TC, TG, LDL, HDL) and liver function (TBIL, TP, ALB, and globulin) tests and some enzymes, which include ALP, AST, ALT, GGT, and CK. The results showed a significant increase in serum glucose, TC, TG, LDL, TBIL, TP, ALB, globulin, ALP, AST, ALT, GGT, and CK with no significant changes HDLin the oral contraceptive users as compared to the control group. In conclusion,using OCs affects lipid profile and liver function compared to the control group.

KEY WORDS: MILLIONS-OF FEMALES, CONTRACEPTIVE PILLS (OCS) LIVER FUNCTION.

INTRODUCTION

Millions-of females worldwide use oral contraceptives pills (OCs) to improve their health outcomes and family planning (Hall and Klein, 2017, Montoya and Bos, 2017). The changing beliefs toward fertility and birth spacing and the rapid change in the socio-demographic pattern in the Saudi Arabian Community have resulted in a notable increase in the use of OCs (Yasmeen et al., 2020). Al-Harazi et al., (2019) conducteda cross-sectional survey through web in all the regions of the kingdom of Saudi Arabia and found that OCs was the first choice (40.3%). Moreover, Alhusain et al., (2018) reportedthat OCs were the most commonly used contraceptive type used (31.8%) inthe Jeddah region of Saudi Arabia. Suleiman(2013) concluded that OCs are obtainable withouta prescription, and pharmacist counseling might sometimes be users' only source of information in Saudi Arabia.

OCs are used to prevent gestation by inhibiting ovulation and implantation(Bawah et al., 2018).Two main types of OCs;Combined Oral Contraceptive pills (COCs) which contain both estrogen and progesterone and, progestogen-only pills (POCs) (Busund et al., 2018). OCs use has been related to an unfavourable influence on lipids

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metabolism byincreasing the level of atherogenic lipids which are strongly associated with cardiovascular disorders (Ferreira et al., 2017a, Elekima and Inokon, 2019). OCs influence on lipid levels dependent on the estrogen dosage respective to the progestin dosage, and androgenic of the progestin (Sirmans and Pate, 2014). Estrogen tends to have beneficial influence by increasing high-density lipoprotein (HDL) and diminishing low-density lipoprotein(LDL).However, triglycerides (TGs) concentration as well increases(Shufelt and Merz, 2009). Progestin appear to have a reverse influence, where, they cause diminishing in HDL levels and rises in LDL(van Rooijen et al., 2002).

Mohammed (2018) observed a correlation between the use of COC and the increase in total cholesterol(TC), LDL-C and the decrease HDL-C in women who used COC. Other studies revealed a significant rise in serum levels of TC, TGs, LDL-C, and decreased HDL-C in the oral contraceptive users compared to the control groups(Mohamed, 2016, Ferreira et al., 2017b). The change in serum lipid profile are key factors in cardiovascular disease (Kazemi et al., 2018).For better estimation and forecasting riskiness of cardiovascular diseases (CVD), many clinical studies have a go at to introduce other markers of atherogenic dyslipidemia, such as coronary risk index (CRI) and atherogenic index (AI) (Ebrahimzadeh et al., 2016). Minahan et al., (2015) reported that CK to be significant higher in OCs users (low estrogen levels) compared to nonusers.

Liver is play a major role in the metabolism of progesterone and oestrogens (Liu and Lebrun, 2006).Estrogens and OCs are both of them associated with several liver-related complications, including intrahepatic cholestasis, hepatic adenomas, sinusoidal dilatation, hepatocellular carcinoma, peliosis hepatis, hepatic venous thrombosis, and a raised risk of gallstones(Diseases, 2012). Kowalska et al., (2018) concluded that OCs could induce alterations in liver enzymes activities and proteins concentration in serum women users OCs.

A study conducted by Boshra (2017) on rats exposed toethinyl estradiol(EE)(100 ugs/kg b.w.) had a significant raising of biochemical markers: AST, ALT, ALP, GGT, and TBIL.Egoro, Tokoni, and Anakwe in (2018) have reported the long-run users of OCs containing a lower dosage of progestin and estrogen composition for a period of ≥ 2 years induced a significant rise in AST, ALT, ALP, and TBIL. Ekhato et al., (2014) observed that administration of lower doses of synthetic OCs to normal rabbits group induced a significant rise in total bilirubin, direct bilirubin, total protein, ALT, and AST compared to non-administrated control rabbits which may impact the liver function and cellular integrity. Therefore, this study aimed to assess the incidence of biochemical changes in lipid profile levels and liver function in Saudi females using OCs.

MATERIALS AND METHODS

Subject and study design: This study was carried out on a total number of 55 Saudi women between ages 20 and 40 years. Participants' were divided into two groupings: 30 women used OCs for a minimum time of one year, and 25 health woman controls. The criteria for inclusion in this study were no evidence of pregnancy and diseases. Lifestyle, physical activity, and medical history information, such as age, use of OCs, duration of OCs use, and OCs type, were obtained through an interview with the subjects. Reproductive history includes the number of pregnancies, age at menarche, parity; characteristic of initial menstrual flow (irregular or regular) was also collected. After the interview, weight, height, blood pressure, glucose was measured, and the (BMI) was estimated.

Blood sample collection: A blood specimen was collected by venipuncture after 10-12h of fasting and put in tubes with a clot activator and serum gel separator. Specimens allowed to clot for two hours at room temperature then centrifuged by for three minutes at 4000 g. The supernatant was collected immediately and stored -24 C.

Biochemical analysis: Serum was used for estimated blood glucose and lipid profile: total cholesterol (TC), triglycerides (TGs), high-density lipoprotein(HDL), and low-density lipoprotein (LDL) levels by colorimetric technique using mercantile Kit.The activity of serum Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), GGT Gamma-glutamyl transferase (GGT), Creatine kinase (CK) and total bilirubin (TBIL), total protein (TP), albumin (ALB), and globulin were also estimated by colorimetric technique using mercantile Kit from SIMENS. Was calculated Atherogenic Index (AI) = (LDLC)/(HDLC), Coronary risk index (CRI)= (TC)/(HDLC) by using the formula suggested by (Kazemi et al., 2018a; Kinosian, Glick, & Garland, 1994), and Cardiovascular risk index (CVRI) = (TG)/(HDLC) was also estimated by (Ramli et al., 2018).

Statistical analysis: The data was analyzed using SPSS. The final results were expressed as mean ± standard deviation (SD). An independent t-test was used to analyze the differences between bath oral contraceptives users and of non-user control. The relationship between variables was ascertained by means of Spearman's correlation coefficient. Values of $P > 0.05$ were considered non significantly different, while those of $P < 0.01$ and $P < 0.05$ were considered highly significant and significant, respectively.

RESULTS AND DISCUSSION

The present study intended to determine the effects of OCs use on biochemical changes included 30 women who had used OCs for at least 12 months and 25 women who had never used OCs. There was no significant difference in the age, height, weight, body mass index (BMI), systolic and diastolic blood pressure, physical activity, and types of OCs between the two groups.

The current results showed that lipid profile levels (TC, TG, LDL) and serum glucose were significantly increased (p = 0.000, 0.027, 0.000, 0.007 respectively) accompanied with non-significant change (p =0.422) in HDL among women using OCs when compared to control group (Table 2).As shown in table (3), there was a highly significant (P=0.000) elevation in the (AI) and (CRI) in women OCs users compared to the control group. However, there was no significant difference (P=0.136) in (CVRI) between the cases and control.

Table 1. Characteristics of the Oral Contraceptive users and non-users

Demographics variables	Mean±SD	Mean±SD	P-vale	Sig
Age (Years)	33.64±6.06	33.866±4.70	0.877	No Sig
Height (m)	1.63±0.049	1.627±0.097	0.873	No Sig
Weight (Kg)	68.88±10.78	63.80±12.63	0.119	No Sig
BMI (Kg/m)	25.79±3.088	24.16±4.80	0.150	No Sig
SBP	122.0±4.25	122.3±11.3	0.87	No Sig
DBP	78.0±4.50	78.2±2.96	0.84	No Sig
Physical activity	N (%)	N (%)		
Yes	4(16.0%)	4(13.3%)		
No	21(84.0%)	26(86.7%)		
Type of OCs	-			
Combined (Gynera)	-	4(13.3%)		
Combined (Yasmine)		17(56.7%)		
Progesterone (Microlot)	-	9(30.0%)		

NOC non-oral contraceptives, OCs oral contraceptives, BMI body mass index, SBP Systolic blood pressure, DBP diastolic blood pressure.
Values are as Mean±SD, p<0.05 significant as compared to the non-users

Table 2. The effect of OCs on lipid profile and glucose level

Variables	Group	N	Mean±SD	P-value	Sig
Glucose	NOC	25	4.3240±0.5125	0.007	Sig
	OCs	30	4.860±0.8810		
Total Cholesterol (mmol/L)	NOC	25	3.2296±0.94734	0.000	H. Sig
	OCs	30	5.2073±0.63847		
Triglycerides (mmol/L)	NOC	25	0.7972±0.36199	0.027	Sig
	OCs	30	1.0953±0.59341		
LDL (mmol/L)	NOC	25	1.8356±0.71385	0.000	H. Sig
	OCs	30	3.2683±0.68374		
HDL (mmol/L)	NOC	25	1.3712±0.28744	0.422	No. Sig
	OCs	30	1.4380±0.31817		

NOC non-oral contraceptives, OCs oral contraceptives, LDL low-density lipoprotein, HDL high-density lipoprotein. Values are as Mean±SD, p<0.05 significant as compared to the non-users

As demonstrated in the table (5), observed a significant increase in serum TBIL, TP, ALB, and globulin levels (P=0.000, 0.000, 0.001, 0.012) between controls and OCs users, respectively. The effect of the duration (12 months and above) of using OCs on all the pervious parameters, showed a statistically significant difference in TC, LDL, Glu, AI, CRI, ALT, AST, ALP, GGT, TB, TP, ALB, and globulin. Also, TG was significantly higher started after five years. However, no significant differences in the CVRI and HDL were noticed (Table 6).

The primary health hazards of OCs are cardiovascular diseases, particularly coronary artery disease, stroke, and venous thromboembolism (Poulter et al., 1996).

OCs adversely effects of lipid profile in women of child bearing age (Faryal,Rashid and Hajra, 2012). Prolonged use of OCs by women during their reproductive age can induce metabolic changes that may contribute to an raised risk of coronary heart (Jamil and Siddiq, 2012). In the current study, there is a significant increase in serum glucosein the OCs users as compared to control group. A similar result was reported by Kofole et al.,(2019). The mechanism through which hormonal contraception causes blood glucose levels to rise has yet to be elucidated (Kofole et al., 2019). One possible mechanism as demonstrated in rats investigated the influence of estradiol on the insulin receptor of ovariectomized rats treated with different hormonal doses that high-doses of estradiol decrease the sensitivity of insulin via the carbohydrate mechanism (González et al., 2002).

Table 3. Comparison of atherogenic indices among OCs users and non-users

Variables	Group	N	Mean±SD	P-value	Sig
AI	NOC	25	1.38±059	0.000	H. Sig
	OCs	30	2.40±0.78		
CRI	NOC	25	2.41±0.81	0.000	H. Sig
	OCs	30	3.80±0.97		
CVRI	NOC	25	0.63±0.37	0.136	N. Sig
	OCs	30	0.84±0.59		

NOC non-oral contraceptives, OCs oral contraceptives. Values are as Mean±SD, p<0.05 significant as compared to the non-users

Table 4. The effects of OCs users on enzymes activity

Variables	Group	N	Mean±SD	P-value	Sig
ALT (U/L)	NOC	25	16.04±2.24	0.000	H. Sig
	OCs	30	26.00±7.268		
AST (U/L)	NOC	25	16.72±4.64	0.000	H. Sig
	OCs	30	25.66±7.40		
ALP (U/L)	NOC	25	59.12±8.67	0.001	H. Sig
	OCs	30	75.60±22.33		
GGT(U/L)	NOC	25	6.48±3.73	0.000	H. Sig
	OCs	30	20.86±12.73		
CK (U/L)	NOC	25	52.84±19.34	0.000	H. Sig
	OCs	30	102.13±51.13		

NOC non-oral contraceptives, OCs oral contraceptives, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma-glutamyl transferase, CK: Creatine kinase. Values are as Mean±SD, p<0.05 significant as compared to the non-users

The present study reported a significant increase in total-cholesterol, triglyceride and LDL and no significant change in HDL in the OCs users as compared to control group. A similar result was reported by Asara et al.,(2014). Giribela et al.,(2015) observed that taking OCs containing estradiol and drospirenone during six months resulted in significant increases in TC and TG levels, but no significant changes in HDL and LDL levels when compared to non-OC users. Other studies revealed a significant increase in serum levels of TC, TG, LDL, and decreased HDL in the OCs users compared to the control group (Mohamed, 2016, Ferreira et al., 2017b, Mohammed, 2018). Kowalska et al., (2018) observed higher TG and lower LDL levels between users and nonusers of OCs with no changes in TC and HDL levels. The increase in total Cholesterol levels among women using oral contraceptive might be due to impaired lipoprotein metabolism and increased in β-lipoprotein cholesterol (Naz et al., 2012).

Triglyceride level was significantly higher among women using OCs. The use of estrogens is associated with increased hepatic synthesis of triglycerides and suppression of hepatic lipase expression, resulting in increased serum levels of triglycerides (Hassan et al., 2014). LDL- cholesterol levels was significantly higher in women using OCs might be because increase lipoprotein synthesis rather than impaired lipolytic catabolism, in association with accumulation of cholesterols as result increased LDL (Yesmin et al., 2013). Estrogen reduces LDL cholesterol levels, and progestin may oppose this effect (Bradley et al., 1978). Combination OCs also may increase serum LDL levels and androgenic steroids appear to cause elevation of LDL levels (Tikkanen and Nikkila, 1986).

Table 5. Serum of total bilirubin, total protein, albumin, and globulin in OCs users and non-users.

Variables	Group	N	Mean±SD	P-value	Sig
TBIL (umol/L)	NOC	25	4.124±2.096	0.000	H. Sig
	OCs	30	7.766±3.692		
TP(g/L)	NOC	25	69.08±12.16	0.000	H. Sig
	OCs	30	82.20±4.57		
ALB(g/L)	NOC	25	34.62±5.146	0.001	H. Sig
	OCs	30	40.60±6.88		
Globulin(g/L)	NOC	25	34.46±12.92	0.012	Sig
	OCs	30	41.59±7.07		

NOC non-oral contraceptives, OCs oral contraceptives, TBIL total bilirubin, TP total protein, ALB albumin values are as Mean±SD, p<0.05 significant as compared to the non-users

Regarding the atherogenic index (AI) and coronary risk index (CRI), there was a significant increase in the OCs users compared to the control group. These results were agreement with Asare et al., (2014). The elevated atherogenic indicators point toward their increased

susceptibility for (CVD) complications (Chakraborty et al., 2019). According to Chait and Eckel (2016), the atherogenesis occurs due to a possible endothelial injury. When there is endothelial injury, macrophages move to the site to clear off oxidized LDL. Macrophages ingest extra lipids and become foam cells, releasing cytokines with the consequent increase in cell proliferation and a further decrease in the diameter of the lumen of blood vessels; this hardens to form a plaque within the lumen (Chait and Eckel, 2016).

Table 6. Effect of duration of OCs use on biochemical parameters

	Control N = 25	Duration of OCs		
		1-2 years n = 7	3-4 years n = 10	5-7 years n = 13
TC	3.230 ± 0.947	4.753± 0.409	4.989 ± 0.529	5.620 ±0.589
		P= 0.000	P= 0.000	P= 0.000
TG	0.797 ± 0.362	1.020 ± 0.501	0.968 ± 0.600	1.234 ±0.646
		P= 0.305	P= 0.661	P= 0.029
LDL	1.836 ± 0.714	3.020 ± 0.614	2.949 ±0.698	3.648 ±0.546
		P= 0.000	P=0.001	P= 0.000
HDL	1.371 ± 0.287	1.210 ± 0.339	1.504 ±0.289	1.510 ±0.291
		P= 0.273	P= 0.235	P= 0.186
Glucose	4.324 ± 0.5126	4.200 ±0.698	4.960 ±0.917	5.139 ±0.810
		P= 0.855	P= 0.012	P= 0.002
AI	1.382 ±0.599	2.678 ± 0.880	2.027 ±0.570	2.552 ±0.829
		P=0.001	P=0.007	P= 0.000
CRI	2.416 ± 0.814	4.234 ± 1.308	3.410 ± 0.597	3.871 ± 0.960
		P=0.001	P=0.001	P= 0.000
CVRI	0.637 ± 0.374	0.955 ± 0.720	0.725 ± 0.607	0.876 ±0.547
		P= 0.261	P= 0.957	P= 0.210
ALT	16.040 ± 2.245	25.286 ±4.889	23.800 ±4.442	28.077 ±9.587
		P= 0.000	P= 0.000	P= 0.000
AST	16.720 ± 4.641	30.00 ±10.646	23.300 ±5.165	25.154 ±6.309
		P= 0.001	P=0.001	P= 0.000
ALP	59.120 ± 8.676	82.429 ±33.221	69.800±12.951	76.385 ±21.724
		P= 0.110	P=0.022	P= 0.009
GGT	6.480 ± 3.732	22.286 ±10.515	21.700 ±16.958	19.462 ±10.814
		P= 0.000	P=0.000	P= 0.000
CK	52.840 ±19.34	145.857 ± 52.740	93.600 ± 46.203	85.154 ± 42.879
		P= 0.000	P=0.002	P=0.009
TBIL	4.124 ± 2.096	7.843 ±3.804	7.730 ±4.798	7.754 ±2.908
		P= 0.013	P=0.022	P= 0.001
TP	69.080±12.165	81.571 ±3.867	82.00±5.185	82.692 ±4.733
		P= 0.000	P= 0.000	P= 0.000
ALB	34.620 ± 5.147	43.071 ±12.840	39.560 ±2.887	40.085 ±4.607
		P= 0.070	P=0.004	P= 0.005
Globulin	34.46 ± 12.926	38.500 ± 12.487	42.440 ± 5.385	42.608 ± 3.740
		P= 0.043	P=0.007	P= 0.001

The values are the mean ± S.D. of parameters measured, Significantly different from control value at P<0.05*, 0.01**, 0.001***

In this study, there was a significant increase in ALT, AST, ALP, and GGT activities in the OCs users compared to the control group. This result was in coincidence to Kowalska et al., (2018) , Toryila et al., (2018) in women and Ekhato et al., (2014) in rabbits. In the study by Al-Fartosi (2017), treated rats with combined oral contraceptive (COC) showed a significant increase in liver enzymes (AST, ALT) levels as compared to the control group. Raised serum level of ALP, AST, and ALT among women using OCs might be because functional

alterations involving the hepatic excretory mechanism (Taneepanichskul,Jaisamrarn and Phupong, 2007). Higher activities of ALT, AST, and GGT in the plasma of OCs users prove the adverse effect of OCs on the liver (Kowalska et al., 2018a).

The results indicated a significant increases in CK in the OCs users as compared to control group, this finding is in agreement with Hicks et al., (2017).Serum CK is an influential early diagnosis of not only myocardial infarction but also any kind of myocardial injury due to it is found in myocardial tissue abundantly, and its virtual absence from most other tissues and its consequent sensitivity(Priscilla & Prince, 2009).Minahan et al., (2015), reported that CK to be significantly higher in OCs users (low estrogen levels) compared to non-users. Estrogen has been reported to decrease cell membrane fluidity; this way, cell membrane stability increases, preventing CK's leakage from the intracellular membrane (Carter,Dobridge and Hackney, 2001). The findings of this study showed a significant increase in serum TBIL, TP and ALB in the OCs users as compared to control group. Our results were in agreement with previous studies (Ekhato et al., 2014, Iyomon et al., 2015, Kowalska et al., 2018b).

Administration of lower doses of synthetic OCs affect on liver functionality by increasing red blood cell destruction which lead to rise of TBIL level. This is suggested considering the function of the liver in filtration, storage, and metabolism of blood and the formation and excretory of bile (Guyton and Hall, 2006). The hyper-proteinaemic impact of OCs indicated by Hall (2015) who suggested that OCs effect on protein metabolism and the colloid osmotic pressure of plasma, muscle devastation, and transport functions via the protein transporting mechanism through ALB. This effect perhaps because the estrogen contained in OCs is known to stimulate the hepatic synthesis of various nutrient-specific transport proteins. Estrogens have long been known to cause intrahepatic cholestasis in susceptible women during pregnancy, during postmenopausal hormone replacement therapy, or after administration of OCs. Estrogen receptor alpha-mediated repression of hepatic transporters and alterations of bile acid biosynthesis may contribute to the development of the estrogen-induced hepatotoxicity (Yamamoto et al., 2006). All hormonal contraceptives contain progestogens. The liver processes progestogens and estrogens differently due to liver cells have estrogen receptors but no progestogen receptors (Sitruk-Ware, 2008).

This study evaluates the effects of duration of OCs use on lipids profile, which is represented by a significant increase in TC, TG, and LDL levels. Also, glucose, AI, and CRI have shown the same results. These results agree with a study by (Sufa,Abebe, and Cheneke, 2019). Similarly, Sultana and Khatun, (2016) have reported serum TC, TG, and LDL were significantly higher among contraceptive users of > 5 years duration than those among five or < 5 years duration. Furthermore, change substantially ($p \leq 0.05$) in ALT, AST, ALP, GGT, CK, TB, TP, ALB, and Globulin in women used OCs for more than one year compared to non-users (control group). However, observed a significant change was in ALP after two years of using OCs. Egoro et al., (2018) demonstrated that elevated levels of plasma ALT, AST, ALP, and TB in long-term users of OCs containing lower doses of estrogen and progestin composition for ≥ two years as compared to(control group). Similarly, significant elevations in GGT and globulin in OCs users after one and two years compared to the non-users (Naz et al., 2016).However, the long-term users of the OCs on HDL and CVRI have shown no significant difference ($p \geq 0.05$) compared to the mean value of non-users of OCs (control group). These results were concord with findings of Bawah et al., (2018).

CONCLUSION

In this study, there was an effect of using OCs on lipid level and liver function compared to the control group. Moreover, there is a statistically significant relationship between the duration of OCs use and the lipid profile. Therefore, the lipid profile must be evaluated while using oral contraceptives and women should be aware of the type of contraception that is appropriate for them.

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The Common Pathological Factors Between Polycystic Ovary Syndrome and COVID-19 Infection: A Review

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a widespread hormone condition that engaged in infertility and metabolic disorders, like diabetes and cardiovascular diseases. The prevalence of PCOS among women of reproductive age ranged from 6% to 10%. There are many pathophysiologic factors associated with PCOS development, including increased blood insulin level, which stimulates the overproduction of androgens. The second important factor is the low-grade inflammations that accompany PCOS condition. In March 2020, the World Health Organization (WHO) has been announced the widespread of coronavirus-2 (SARS-CoV-2) disease (COVID-19) as a pandemic. The researchers documented the presence of certain diseases as risk factors for increased COVID-19 infection and severity including diabetes, hypertension, and obesity. This study aims to review PCOS's comorbid conditions that can predispose to increased risk of acquiring COVID-19 infection or magnifying its complications or even causing death. Studies have indicated that women with PCOS have many factors and pathologies that greatly increase the incidence of complications of COVID-19. These factors include excessive androgen production, change in microbiome formation, obesity, insulin resistance, vitamin D deficiency, and NAFLD. These factors cause decreased immunity, increased inflammatory reactions, and increased expression of the ACE2 (the gate that enables the virus to penetrate the cells). Therefore, it is necessary to inform PCOS women in order to increase precautionary measures. These women with complicated health conditions should receive high-level health care.

KEY WORDS: POLYCYSTIC OVARY SYNDROME; COVID-19; ANDROGEN; MICROBIOME; INSULIN RESISTANCE..

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a widespread hormonal health problem which is engaged in infertility and metabolic disorders, like diabetes and cardiovascular diseases, (Sam, 2007). The prevalence of PCOS among women of reproductive age ranged from 6% to 10%. PCOS's main characteristics are related to increased androgen production; these constitute oligo and amenorrhoea, impaired fertility, hirsutism, acne, and alopecia (Sam and Dunaif, 2003; Sam, 2007).Besides the severe reproductive consequences, several metabolic features accompany PCOS incorporating insulin resistance; the troubles that cause enhanced risk for glucose intolerance, and insulin independent diabetes, (Kyrou et al., 2000, 2015; Möhlig et al., 2006; Randeva et al., 2012; Pasquali, 2018, Barber et al., 2019; Manisha et al., 2020).

Obesity is a prevalent feature in women with PCOS, as nearly 40% to 80% of women with this disorder are observed to be overweight or obese(Sam, 2007).There are many pathophysiologic factors associated with PCOS development, including increased blood insulin level,

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which stimulates the overproduction of androgens. The second important factor is the low-grade inflammations that accompany PCOS condition. Studies have reported that women with low-grade inflammation may experience PCOS(Hignett et al., 2011). Genes likewise is a PCOS predisposing factor. The syndrome usually affects mothers, daughters, and sisters in the same family, (Urbanek, 2007).

Finally, the immoderate exposure of fetuses to androgens can permanently inhibit normal genes function. The androgens can boost lipid distribution in the abdominal region in a male model pattern, which promotes insulin resistance and low-grade inflammation(Hignett et al., 2011).Renin-angiotensin system (RAS) is an important system that regulates both cardiovascular and kidney function(Unger, 2002; Vejakama et al., 2012). An early study has documented that RAS is linked to hormonal changes and insulin resistance, (Liu, 2007). There is increasing proof that RAS is enhanced in PCOS patients, which may contribute to the overstimulation of the ovary and excess androgen production (Moin et al., 2020).

In March 2020, the World Health Organization (WHO) has been announced the widespread of coronavirus-2 (SARS-CoV-2) disease (COVID-19) as a pandemic (Cucinotta and Vanelli, 2020).Even though the majority of COVID-19 patients are either asymptomatic or with mild symptoms, many others face severe disease with increased mortality (Yuki et al., 2020).A growing body of scientific proof elucidated that the prevalence of serious COVID-19 is remarkably elevated in old versus youth and males versus females (Cai, 2020; Docherty et al., 2020; Guan et al., 2020; Guo et al., 2020; Jin et al., 2020; La Vignera et al., 2020; Wu and McGoogan, 2020). The researchers documented the presence of certain diseases as risk factors for increased COVID-19 infection and severity.

These include diabetes and hypertension. Obesity has also been listed as a risk factor for corona virus infection(Bornstein et al., 2020; Guan et al., 2020; Li et al., 2020).Of particular attention is that the angiotensin-converting enzyme-2 (ACE2) has been utilized by COVID-19 to enter the host target cells and, therefore, significantly impact the RAS pathway(Wiese et al., 2020). Indeed, it is obvious that many risk factors are overlapping between PCOS women and COVID-19 susceptibility. Hence, it may be proposed that the PCOS women are potentially at great than anticipated risk if challenged with a COVID-19 virus infection. This study aims to review PCOS's comorbid conditions that can predispose to increased risk of acquiring COVID-19 infection or magnifying its complications or even causing death.

Overproduction Of Androgens: In PCOS, up to 60 % of androgens are released by the ovaries, whereas the adrenal gland provides the residual 40%. It is known that the fundamental cause of increased androgen production in women with PCOS are androgens from both the ovary as well as the adrenal gland (Cedars et al., 1992).In PCOS females, low concentrations of sex-hormone-binding globulin (SHBG) often lead to elevated serum free androgen. As confirmed in several studies, SHBG concentrations are inversely correlated with serum insulin concentrations or with the extent of insulin sensitivity in females both with and without PCOS. In addition, decreasing insulin secretion in PCOS obese females without affecting the insulin resistance is accompanied by increased serum SHBG concentration (Nestler et al., 1991).

Sex hormones are responsible for the immune response, as estrogen is known to improve immunity, whereas testosterone is known to inhibit it (Strope et al., 2020). Besides, androgens control an essential protease engaged in viral entry, TMPRSS2 (Hägglöf et al., 2014). The experimental studies provided evidence that sex hormones increase the expression and activity of ACE-2 in different tissues, including the cardiac, renal, and adipose tissue(La Vignera et al., 2020).The role of androgen receptor (AR) gene polymorphisms in the development and progression of cardiac complications and hypertension in COVID-19 infected male subjects cannot be ignored because the expression of ACE2 in the cardiac muscle is modulated by the androgens(Dalpiaz et al., 2015).

It has recently been explored that a high prevalence of male pattern baldness (often associated with increased serum androgen) in the hospitalized COVID-19 patients, potentially indicating that androgens could be involved in the incidence of COVID-19(Goren et al., 2020). Therefore, a possible correlation between androgens and the acuteness of COVID-19 seems probable (Goren et al., 2020; McCoy et al., 2020; Wambier and Goren, 2020; Wambier et al., 2020) and may further suggest the hypothesis that PCOS may constitute an additional potential risk for the severity of COVID-19.This assumption is indeed essential because females with PCOS either manifest hyperandrogenism (androgenic alopecia) or under therapy with anti-androgen (spironolactone or finasteride)(Quinn et al., 2014; Kyrou et al., 2015; Teede et al., 2018).

Against this hypothesis, a retrospective cohort study that constitutes forty-five COVID-19 patients at the intensive Care Unit at the University Hospital Hamburg-Eppendorf, Germany, documented that severely COVID-19 diseased men (n=35) showed a severe decline in their serum testosterone and dihydrotestosterone levels. In contrast, the women (n=10) showed increased serum testosterone concentration unaccompanied by any alterations in dihydrotestosterone concentration (Schroeder et al., 2020). Furthermore, in a study of 31 Italian hospital-admitted male patients, a significant gradual decrease in both serum-free and total androgen concentrations was substantially linked with the need for special respiratory care and intensive care (Rastrelli et al., 2020).

Perhaps more research will be required to validate the correlation between the amount of serum androgens and the seriousness of COVID-19 infection (Kyrou et al.,

2020). Many studies have shown that males are more vulnerable to coronavirus hazards and complications than females. And this was due to the increase in males' testosterone hormone levels. Females with PCOS who have raised serum testosterone concentration can also be at higher risk for complications of COVID-19.

Microbiome Composition: The results of Torres et al. (2018) showed that females with PCOS have fewer different strains of intestinal microbiome, a finding that seems to be correlated with increased serum concentrations of testosterone. In their study, the researchers analyzed 73 faecal swabs ofPCOS females. Their samples were matched with swabs from 48 women with no PCOS and 42 women with polycystic ovaries, but with no other PCOS characteristics. The study results showed that females with PCOS had the minimal diverse intestinal bacteria, females without the disease had the maximum diverse intestinal bacteria, and females with polycystic ovaries have diversity in the intestinal microbes than females with PCOS. The researchers indicate that testosterone and other androgens can help form the intestinal microbiome, and these alterations can impact the quality of life of PCOS females.

It has been shown that the gut microbiome modifies the immune system, which helps defend against foreign pathogens either by immunity or by competitive exclusion (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Kamada et al., 2013).The normal microflora stimulates interleukins generation in the gut that is defensive against pathogens (Franchi et al., 2012).Not only are the impacts of commensals local, but they can be systemic. The decline in intestinal microbiota owing to antibiotics is consistent with impaired activity of T and B cells versus intranasal influenza(Ichinohe et al., 2011).By rectal administration of toll-like receptor (TLR) agonists, defensive immunity against intranasal influenza is restored, leading to the formation of IL 1-β and IL 18 (Brugger et al., 2016).Scientists have found that citizens in developing countries have a lower mortality rate during COVID-19 relative to developed nations. And the reason for that, scientists have proposed, is the exposure of the inhabitants of these countries to a high microbial load, increasing immunity. Scientists have shown that the richness of the microbiome has a protective effect against external infections, like, COVID-19 (Kumar and Chander, 2020).

In a pilot study including 15 COVID-19 patients, the researchers observed persistent changes compared to controls in the faecal microbiome during the hospitalization period. Alterations of the faecal microbiota were parallel with COVID-19 riskiness levels, (Zuo et al., 2020).A new Wuhan, China, the analysis found a correlation between both the composition of the intestinal microbiota and the susceptibility of healthy subjects to COVID-19 (Gou et al., 2020).The presence of Lactobacillus species in the intestine enhances the production of one of the most important anti-inflammatory cytokines, IL-10, and this is what makes the scientists expect the best with corona treatment. High amounts of pro-inflammatory species bacteria, comprising Klebsiella, Streptococcus, and Ruminococcus gnavus, associated with greater amount of pro-inflammatory cytokines and enhanced complications of illness. These bacteria have been described to be abundant in the proinflammatory gastrointestinal environment of people who suffer from a lot of diseases, such as, diabetes, obesity, irritable bowel disease, and hypertension(van der Lelie and Taghavi, 2020).

The concentration of ACE2, which is the target of the COVID-19 virus, was also increased in the dysbiotic gut environment (Chan et al., 2020). As PCOS is linked with obesity, hyperglycemia, and increased blood pressure, therefore, PCOS females may have the same alterations in the composition of the intestinal microbiome caused by these illnesses and hence they will suffer the severe complications of COVID-19.Women with PCOS have a dysbiotic gut microbiome as well as variation in microbial composition (Morgante et al., 2020).

Obesity And Insulin Resistance: Growing numbers of research have proposed that pro-inflammatory cytokines are implicated in the pathophysiology of PCOS, which is also marked by the existence of low-grade chronic inflammation. In females with PCOS, many inflammatory cytokines have been identified to be linked with insulin resistance (Al-Musawy et al., 2018). The study's findings indicated that high amounts of interleukin-6 (IL-6) in PCOS females were positively correlated with the ratio of homeostasis model assessment of insulin resistance (HOMA-IR) and total testosterone ratio in both slim and overweight PCOS females (Peng et al., 2016). The majority of females with PCOS are also obese and visceral fat is included in the presence of proinflammatory mediators observed in PCOS women (Sepilian and Nagamani, 2005).

It has been well known that females with PCOS and obesity are exhibiting significant impairment of fat tissue function and overactive secretion of adipokine/cytokine including elevated secretion of IL-6, tumor necrosis factor-α (TNF-α), and leptin, resulting in a prolonged pro-inflammatory condition (Kyrou et al., 2015, 2018). Besides, females with PCOS often have polymorphisms in gene expressing pro-inflammatory cytokines, like TNF-α and IL-6, compared to normal females (Guo et al., 2015; Zhang et al., 2020).IL-6 is an inflammation promoter that regulates the release of many cytokines in females with PCOS (Vural et al., 2010). It controls many ovarian functions including ovulation, conception, and implantation. In PCOS women, serum and granulosa cell IL-6 levels are increased (Lee et al., 2017; Al-Musawy et al., 2018) and studies have confirmed that elevated IL-6 could be correlated with PCOS insulin resistance and hyper androgenism (González et al., 2012).

Preliminary findings from the UK (Intensive Care National Audit & Research Centre, 2020), China (Peng et al., 2020), and the USA (Petrilli et al., 2020) hospitals indicate that obese COVID-19 patients have a poorer prognosis. It is abundantly clear that there are specific

mechanisms through which obesity and its consequences such as metabolic and inflammatory alterations, deteriorate the outcome of COVID-19 (Finucane and Davenport, 2020).The initial findings proposed that people with complicatedCOVID-19 appear to be aged men with high blood pressure, hyperglycemia, and increased serum liver enzymes all increase the probability that insulin resistance may exerted an essential function in mediating COVID-19 complications(Finucane and Davenport, 2020).

Studies have indicated that the seriousness of COVID-19 may be linked with the predisposition to release inflammatory cytokines (cytokines storm syndrome) including various inflammatory interleukins, like TNF-α, IL-6, and IL-1β in the patient's lung tissue (Fagone et al., 2020; Mehta et al., 2020).Evidence shows that in a subset of patients with extreme COVID-19 infection, this syndrome can cause self-sustaining hyper-inflammatory responses, priming respiratory, and multiple organ failure (Fagone et al., 2020).Therefore, there may be a link between the cytokine storm syndrome associated with the risk of COVID-19 and the diseases associated with increased release of proinflammatory mediators, including PCOS. To confirm this hypothesis, many studies are needed.

There is another link between PCOS and the risk of experience serious COVID-19 infection, which is also connectedwith obesity and insulin resistance, which is the increased expression of ACE2 (Morgante et al., 2020).Insulin resistance is often reflected in elevated serum insulin levels (Kahn, 2003).A broad "phenome-wide" Mendelian Randomization research reported that the significant lung ACE2 expression is correlated with many diabetes-related features (Rao et al., 2020).PCOS may be a factor that determines the severity of infection with the COVID-19 virus, due to the accompanying obesity and insulin resistance (Frisardi, 2020), the factors that increase the start of the cytokine storm and the consequent inflammation (Fagone et al., 2020), as well as the increased expression of the ACE2 (Frisardi, 2020), which acts as a receptor for the COVID-19 virus to enter the cells.

Vitamin D Level: Several studies have reported on the relationship between vitamin D deficiency and the severity of COVID-19 infection, as studies have linked the rapid spread of the pandemic in Europeespecially Italy, France, Spain, and England to the emergence of the pandemic following the winter and the consequent lack of exposure to sunlight and vitamin D deficiency (Grant et al., 2020; Marik et al., 2020; Panarese and Shahini, 2020; Rhodes et al., 2020).It has also been reported in several studies that vitamin D deficiency is one of the causes of acute respiratory distress syndrome, besides research has also confirmed an increase in COVID-19 deaths among the elderly and patients with metabolic heart diseases, which also coincides with low levels of vitamin D (Grant et al., 2020; Marik et al., 2020; Panarese and Shahini, 2020; Rhodes et al., 2020).Of interest, the elderly people of Italy and Spain, which were between the key epicenters of the COVID-19 outbreak in Europe, recorded especially deficiency of vitamin D(Ilie et al., 2020).

Vitamin D is a famous cytoprotective hormone that influences the innate and adaptive immune reaction, regulates the activity of IL-6, and inhibits the release of pro-inflammatory cytokines from macrophages and respiratory epithelial cells in response to different viruses(Grant et al., 2020; Marik et al., 2020; Silberstein, 2020; Tian and Rong, 2020).Growing results confirm a negative relation among vitamin D and the incidence of multiple manifestations of PCOS, particularly androgen excess, fertility problems, resistance to insulin, and cardio-metabolic disorder(Muscogiuri et al., 2014; Reis et al., 2017).Furthermore, a meta-analysis study indicates that vitamin D supplementation will effectively decrease the serum concentration of total testosterone and C-reactive protein in females with PCOS, although it increases the levels of antioxidant molecules (Azadi-Yazdi et al., 2017; Akbari et al., 2018).From these studies, it can be concluded that women with PCOS may be more susceptible to complications of COVID-19 due to their vitamin D deficiency, which worsens when quarantine and not exposed to the sunlight (Kyrou et al., 2020).

Non-Alcoholic Fatty Liver Disease: Proofs from clinical studies and meta-analyses suggest a high incidence of non-alcoholic fatty liver disease (NAFLD) in females with PCOS, 34% - 70%, compared to 14% - 34% in normal females (Vassilatou et al., 2010; Macut et al., 2016; Wu et al., 2018).Two possible pathophysiological relations between NAFLD and PCOS are insulin resistance and hyperandrogenism. Insulin resistance appears to interact with obesity and hyperandrogenism, thereby impacting NAFLD and PCOS and being impacted by them (Paschou et al., 2020).A recent Cross-sectional study including 98 Mexican women with PCOS at reproductive age (18-44 years) showed that NAFLD was significantly increased in PCOS women than the normal control women at 69.3% versus 34.6%, respectively. Severe steatosis was the most frequent NAFLD stage between PCOS women (Salva-Pastor et al., 2020).

A retrospective longitudinal cohort study assessing NAFLD rates in 63,120 women with PCOS, using a broad primary care database in the United Kingdom, reported that females with PCOS had an elevated NAFLD rate. Besides, an elevated risk of NAFLD was linked to increased serum testosterone (Kumarendran et al., 2018). Females with PCOS had an increased risk of NAFLD, central obesity, hyperlipidemia, insulin resistance, and metabolic syndrome (Kumarendran et al., 2018).

For COVID-19 patients, NAFLD is a major trigger for hospital admission compared to age, sex, obesity, or other coexisting health problems(Bramante et al., 2020). (Bramante et al., 2020) also showed that by managing NAFLD the risk of hospitalization decreased with obesity. The study also suggests the prominent influence of visceral adiposity in COVID-19 pathophysiology, that enhances the prolonged inflammation and clot formation

provoked by NAFLD (Bramante et al., 2020). The most important risk causes for bad results duringCOVID-19 infection prove to be obesity and metabolic disorder (Yang et al., 2020).

NAFLD is evidence of increased visceral fats, progressive metabolic disorder, and prolonged inflammation (Sheka et al., 2020).Although low expression of ACE2 was normally found in cholangiocytes and hepatocytes, high expression was associated with experimentally induced chronic liver injury and NAFLD (Paizis et al., 2005). Since ACE2 is the way COVID-19 enters the cells, liver injury can lead to increased viral load and worsening outcomes of COVID-19 (Prins and Olinga, 2020; Xu et al., 2020). The hypothesis can be adopted as the women with PCOS being among the most likely to have NAFLD and hence they will also the most likely to have a worse COVID-19 condition.

CONCLUSION

Studies have indicated that women with PCOS have many factors and pathologies that greatly increase the incidence of complications of COVID-19. These factors include excessive androgen production, change in microbiome formation, obesity, insulin resistance, vitamin D deficiency, and NAFLD. These factors cause decreased immunity, increased inflammatory reactions, and increased expression of the ACE2 (the gate that enables the virus to penetrate the cells). Therefore, it is necessary to inform PCOS women to increase precautionary measures. These women with complicated health conditions should receive high-level health care.

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Biomedical Properties of Metal Nanoparticles for Cancer Therapeutics and Management

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ABSTRACT

The nanoparticles (NPs) drawn more interest as they fill the gap between bulk materials and atomic, molecular, bio-molecular or cellular structures. The fabrication of novel metal NPs is a demanding area of research because they display distinctive properties, different from those of bulk counterparts. Because of stability, oxidation resistance metal NPs find wide applications in different areas. For each unique application, NPs of different size and shape are mandatory, therefore several protocols for the preparations of NPs are required. However, other methods such as chemical and physical methods may fruitfully generate pure and well-defined nanoparticles, these are expensive and potentially hazardous to the environment. The use of biological agents can be an alternative to chemical and physical agents for the production of NPs in an eco-friendly manner. In the past decade, plants, algae, fungi, bacteria, viruses and enzymes have been used for the production of low-cost, energy-efficient and nontoxic metallic nanoparticles. Metallic NPs have wide applications in many areas such as engineering, biosensors, catalysis, biomedical and drug delivery. The smaller size of the NPs allows readily interaction with biological system while the material compositions of NPs gives stability and specificity which are important for drug delivery and biocompatibility. In addition, anticipation for development in personalized treatment and management so it make possible to develop and manage the suitable drug. Presently, the utmost area of nanomedicine is the improvement and use of NPs for drug delivery in cancer. NPs are engineered so that they are appealed to cancer cells, which leads to direct treatment of those cells. This methodology decreases damage to healthy cells in the body and also allows for earlier detection of cancer.

KEY WORDS: METAL NANOPARTICLES, SILVER, GOLD, CANCER, BIOMEDICAL.

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INTRODUCTION

Novel metal nanoparticles show outstanding unique physiochemical and biological characteristics. Noble metal nanoparticles can be used in different areas such as biosensor, catalysis, antibacterial, anticancer, antimalarial and antiviral (Marin et al., 2015). In the present decade, fabrication of novel metal nanoparticles can be achieved via various procedures (Mousavi et al., 2016). Although, chemical based methods are the widely applied methods for the fabrication of nanostructure. Though, chemical processes cannot prevent the use or generation of by-product of hazardous chemicals (Celik et al., 2017). However, metallic nanoparticles are extensively used for medical biotechnology including human contacting areas.

Therefore, there is an increasing demand to develop environmentally sustainable methods of nanoparticle fabrication without uses of toxic ingredients (Saratale et al., 2018, Iravani et al., 2020). Among several metal nanoparticles, silver and gold, have been studied and are useful in different fields such as antimicrobials, biosensors, drug and gene delivery etc. In addition, Ag and Au nanoparticles have a unique surface plasmon resonance (SPR) absorption in the UV-vis region. However, now a days bimetallic and trimetallic nanoparticles have also used due to unique properties for biomedical applications such as cancer treatment. (Ali et al., 2020, Ravi et al., 2020) There are different types of metal nanoparticles are described below:

Based on morphology: NPs can also be classified on the basis of their morphologies such as aspect ratio and sphericity. The high aspect ratio nanomaterials of various design such as nano-sphere and nanocubes having low aspect ratio while nanowires having high aspect ratio (You et al., 2017, Singh et al., 2020).

Based on constituents and structures: NPs are either of single or multiple moieties, they may be of organic-inorganic hybrid nature (more than one constituent). The structures of the NPs depend on the nature of these constituents, which are used as reaction precursors during their preparation. Based on nature of constituents, NPs can also be classified as:

Organic NPs: Organic moieties such as lipid, polymers and natural/synthetic organic materials are covered in the class of organic NPs (ONPs) (Rizwan et al., 2017). They are applied in the form of liposomes, metal organic frameworks, polymeric micelles of polyacrylate, polycarbonate, polyester etc. (Zakharova et al., 2017). There are many FDA approved ONPs, which are used as controlled release agents to deliver the desired moiety to the target site. For instance, leuprorelin acetate (synthetic gonadotropin release hormone, GnRH) depot releases in 6 months, is used for the cure of prostate cancer (Hebenstreit et al.,2020).

Polymeric NPs: The polymeric nanoparticles (PNPs) are categorized as colloidal particles of nano scale, which are derived from various polymers including natural, semisynthetic and synthetic polymers (Mohammadi et al., 2020). Some of these NPs are pH and temperature sensitive and prepared from biocompatible and biodegradable polymers.

Dendrimers: Dendrimers are three dimensions globular molecules, mono-dispersed with repeating units having large number of functional groups present on their surfaces. The word “dendrimer” was elicited from Greek word “dendron” that symbolizes a tree (Sapra et al., 2019). They are highly dense and less viscous due to their extensive branching (Patel et al., 2020).

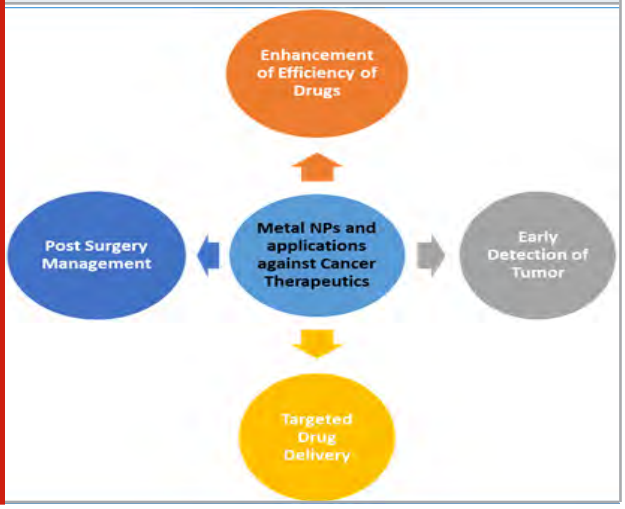
Liposomes: Liposomes are colloidal nanomaterial having vesicular structure. Liposomes are usually made up of phospholipids while few are made up of phosphatidylcholine (Daraee et al., 2016). In liposomes, the double phospholipid layers have an internal aqueous cavity. They are amphiphilic nature and exhibits biocompatibility (Ding et al., 2020).

Inorganic NPs: Inorganic NPs are characterized as particles of nano-sized prepared by the applications of inorganic moieties like metalloid and metal (Tabesh et al., 2017). They possess specific and unique properties, which mainly depend on their sizes. Special properties of inorganic NPs such as versatile physical, chemical, optical and magnetic makes them available for various applications in water treatment to biomedical (Zhao et al., 2018). NPs like gold and silver have strong absorbance and high electron density and particles like iron oxide has great magnetic characteristics. From literature, it can be concluded that Metallic NPs are particularly, prepared at optimum temperature, have no effect on changing the pH and are non-toxic to healthy cells (Yilmaz et al., 2018). Moreover, the novel inventions of magnetic metallic NPs introduced them in environmental applications. (Ravi et al.2019) The use of magnetic NPs particularly in adsorption technology may promise to advance the technique through the post adsorption magnetic separation of adsorbents (Arabkhani et al.,2020). All these features of metallic NPs are size dependent and in many cases, they are considered as best materials. These metallic NPs can be classified as:

Zerovalent (ZV) metallic NPs: ZV Metallic NPs have significant importance due to their distinguished properties in nanoscale compared to their bulk state. They are employed in several applications such as sensing, catalysis, adsorption and imaging due to their unique optical property, sensitivity and larger surface area along with significant photo-stability (Hai et al., 2018). Several methods are employed for the synthesis of these NPs, such as chemical, biological, electrochemical, photolytic, etc. Among them, the most significant and widely used synthetic method is the wet chemical method (chemical precipitation or chemical reduction) and biological. ZV Metallic NPs are synthesized in their uncharged state (zero-valent) through the reduction of their metal precursor using a common reducing agent

i.e., chemical reduction and/or plant extraction method (Reverberi et al., 2018).

Figure 1: Graphical representation of metal nanoparticles and their different applications against various cancer types.



The ZV metallic NP appears different in different conditions, e.g. ZV gold NPs in bulk state is yellow but its aqueous dispersion in nanoscale ranges from red or violet. Similarly, ZV silver NPs looks yellow in their aqueous nanoscale formulation while ZV iron NPs appear in greenish color under aerobic conditions. These changes in appearance are mainly attributed to the mutual oscillations of the electrons in conduction band caused due to the light exposure of a specific wavelength and power. This is a well-known phenomenon termed as localized surface plasmon resonance (LSPR), which occurs due to the collective oscillations of electrons in an externally applied electric field (Feng et al., 2020).

Metal NPs as cancer treatment agent: Cancer is considered as one of the biggest challenges faced by the medical researchers in recent time. The high cost and strong side effects of available options limits their applications for cancer treatment. Further, the timely diagnosis and prognosis of cancer is still a difficult task (Mishra et al., 2012, Restifo et al., 2016) Moreover, the approaches usually used for cancer treatment such as surgery, radiation and chemotherapy have considerable harmful effects (Gill et al., 2014). Thus, there is a crucial need for application of new feasible and novel methods (non-invasive and minimally invasive) for the early diagnosis and therapy of cancers (Li et al., 2015). In this regard, bimetallic nanoparticles (BNPs) and trimetallic nanoparticles (TNPs) have come up as a potential candidate for the cancer therapeutics, which has given a new field of research in the form of cancer nanomedicine. BNPs and TNPs provide the basis for the continuous and targeted release of the anticancer agent at a pace and at a venue to overcome the issues of standard diagnostic and therapeutic methods (Senapati et al.,2018, Sivamaruthi et al., 2019). Fig 1. shows illustration of applications of different metal nanoparticles against various cancers.

Table 1. Anticancer Applications of Metal Nanoparticles

S.N.	Name of Metal Nanoparticles	Size of Nanoparticles (nm)	Application against Cancer Cells/ Tumors	Ref.
1	Silver (Ag)	4.99-25.83	MCF-7	(Jhang et al., 2016)
2	Silver (Ag)	22–85	MCF-7, PC-3, A549, HCT-116	(Abd-Elnaby et al., 2016)
3	Silver (Ag)	10-80	HepG2, A549	(Rajeshkumar et al., 2016)
4	Silver (Ag)			
	Gold (Au)	24-150	HCT-116	(Kuppusamy et al., 2016)
5	Gold (Au)	50-100	HT-29	(Bai et al., 2018)
6	Gold (Au)	10-40	HeLa	(Patil et al., 2019)
7	Silver (Ag)	10-20	DU154, A549, MCF-7, A431	(Singh et al., 2020)
8	Silver (Ag)	24-54	MCF-7	(Kiran et al., 2020)
9	Gold (Au)	5-10	MCF-7	(Munawer et al., 2020)
10	Silver (Ag)	15.45	MCF-7	(Nawaz et al., 2020)

On this account, several researchers have made their effort to synthesize BNP and TNPs also investigated their cancer therapeutic activities. For instance, wang et al. have reported rapid and single-pot approach for the synthesis of Cu/Au/Pt TNPs. The TNPs showed high catalytic behaviour and strong plasmonic absorption in the NIR-I bio window (650-950 nm). Because of these properties, the synthesized Cu/Au/Pt TNPs was used for the application in biosensing and cancer theranostics. The trimetallic nanoparticles have also been used for dye removal and cancer treatment. For instance, Basavegowda et al. (2017) have synthesized trimetallic Fe-Ag-Pt nanoparticles via ultra-sonication. These trimetallic alloys nanostructures have exhibited

excellent catalytic efficiency and have also been used for other applications. Ahmad et al. (2019) formulated trimetallic.

Au/Pt/Ag nanoparticle based nanofluids by green microwave assisted successive chemical reduction method and used this to check the antibacterial activity and compared to these of monometallic Au and bimetallic Au/Pt nanofluid. Liang et al. (2018) immobilized trimetallic Cu-Ni-Co nanoparticles onto the pores of the metal-organic framework by a simple but unique solvent evaporation approach and compared to their bimetallic and monometallic equivalents.

These investigations revealed that the Cu-Ni-Co trimetallic catalysts display superior catalytic activity. Recently, Gu et al. (2019) have synthesized carbon dots embedded bimetallic ZrHf-based metal-organic framework (CDs@ZrHf-MOF). The synthesized bimetallic NP was used to assess to differentiate human epidermal growth factor receptor-2 (HER2) and living HER2-overexpressed MCF-7 cells. Ma et al. (2015) fabricated extremely sensitive electrochemical immune sensor for the sensing of bladder cancer biomarker i.e. nuclear matrix protein 22 (NMP22). This sensor contain reduced graphene oxide–tetraethylene pentamine (rGO–TEPA) and trimetallic AuPdPt NPs. Sharma et al. (2018) have designed Zr2Ni1Cu7 trimetallic nanoparticle and its composite with Si3N4. Both the composite and TNPs were subjected to photo-degradation of methylene blue under visible light. Pradhan et al. (2007) have reported the synthesis and characterization of manganese ferrite based magnetic liposomes. The synthesized materials was further applied for hyperthermia cancer treatment.

CONCLUSION

Thus, based on above literature survey, it can be concluded that the metal NPs could be used as anticancer agent. However, the scant work has been reported on their applications in cancer treatment. Tireless efforts are being made to use metal NPs in the field of cancer management, and further, it was observed that metal NPs have an enormous capability to improve the efficacy of cancer therapeutics. In addition, animal model and clinical human trials are necessary part for the practical applications for these nanoparticles.

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Root and Canal Morphology of Permanent Maxillary and Mandibular Incisor Teeth: A Systematic Review and Comparison with Saudi Arabian Population

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ABSTRACT

Here we summarize the original studies and case reports addressing the root and root canal morphology of permanent anterior teeth among the Saudi Arabian population, comparing findings to the international literature. The maxillary and mandibular central and lateral incisors are among the most likely teeth to require endodontic treatments, so their morphology should be considered for root canal treatment success. All related literature published between 1980 and 2020 in peer-reviewed journals were included in this review. A systematic literature exploration was carried-out using the PubMed, ScienceDirect, Scopus, Evidence-Based Dentistry Journal, and Dental Practice databases. The search terms used were: "root canal morphology", "root morphology", "case report for anterior maxillary and mandibular teeth", and "Saudi Arabian population". Twenty-nine original research articles were identified. Most of the studies used the cone beam computed tomography (CBCT) technique. A total of 29 original research studies were included in this review. In the Saudi-based original research, three studies addressed mandibular and one study maxillary teeth and were conducted in various cities. Twenty-nine clinical case reports are presented: among these, three were Saudi patients. When comparing Saudi data to data gathered in other populations, the findings were mostly consistent in canal and root configuration of maxillary and mandibular anterior teeth. New devices and technologies are clinically useful in the identification of morphological variations in permanent teeth. Greater attention should be given to detecting additional canals. Variation among canals of mandibular anterior and maxillary teeth should be considered for successful endodontic treatment.

KEY WORDS: CANAL CONFIGURATION, CASE REPORT, MANDIBULAR TEETH, MAXILLARY TEETH, ROOT CANAL SYSTEM, MORPHOLOGY, SAUDI ARABIA.

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INTRODUCTION

All root canal treatments (RCTs) rely on knowledge of the tooth morphology, and three-dimensional imaging of root canal systems (Castellucci., 2015; Bansal et al., 2018). It has been reported that the shape and number of roots and canals differ among genders and populations (Al-Fouzan et al., 2012; Mirhosseini et al., 2017; Saati et al., 2018; Mashyakhy., 2019; Valenti-Obino et al., 2019;

Baxter et al., 2020; Ghabbani et al., 2020). Therefore, it is important to be familiar with differences in the tooth outlines and characteristic features among ethnicities. Such knowledge can aid in the location and negotiation of canals (Arslan et al., 2015; Zhengyan et al., 2016; Verma et al., 2017; Martins et al., 2018; Martínez et al., 2018; Popovic et al., 2018; Shemesh et al.,2018).

A study classified the cross-sectional root canal configurations of mandibular teeth as round, oval, long oval, flattened, or irregular (Castellucci 2015). Whoever the majority of permanent of maxillary or mandibular incisors have one root and one canal; however, a small percentage may have a second canal, lateral canal, or apical deltas (Saati et al., 2018). The root canal systems of incisors do not consist of a single canal running uniformly from the orifice of the pulp champers to the apex. In fact, the root canal systems of incisors can be complex due to the splitting and reunion of the canals during its passage to the end of the roots (Vertucci., 1984; Saini et al., 1990; Altunsoy et al., 2014; Razumova et al., 2018; Mashyakhy and Gambarini., 2019; Pan et al., 2019).

The root canal systems of incisors open apically into the periodontium through apical and lateral foramens. During its passage, the root canal presents a variety of configurations, differing among tooth types, genders, and populations (Mirhosseini et al., 2017; Martínez et al., 2018; Popovic et al., 2018; Mashyakhy and Gambarini., 2019). Recently, Neelakantan et al (2010) and Przesmycka and Tomczyk (2016) have compared the efficacy of various methods for visualizing the canal and root morphology. These authors concluded that CBCT is accurate, high-resolution, and can be useful for detailed quantitative and qualitative descriptions of the RC anatomy, (Neelakantan et al., 2010; Filpo–Perez et al., 2015; Przesmycka and Tomczyk., 2016; Martinsa et al., 2020).

Some reviews have noted high global rates of a second canal in the anterior central and lateral incisors (20.4% and 25.3%), respectively (Martinsa et al., 2020), while some studies have reported greatly varying numbers of canals and canal types among the same teeth (Ahmad., 2015; Ahmed and Hashem., 2016; Bansal et al., 2018; Martins et al., 2019). All the published reviews that we identified have noted that knowledge of these preoperative variables could help clinicians anticipate more complex RC anatomic configurations, thus minimizing the possibility of lost canals during treatment. Investigators use various morphological characteristics to classify root canal systems e.g., the number of canals from orifice to apex, the sum of roots and number of canals in each root, or the number of isthmuses (Vertucci., 1984; Bansal et al., 2018), but the most widely used is Vertucci's classification, which classifies root canal systems into eight types.

Variations in the morphology of the canals and roots of maxillary and mandibular central or lateral incisor teeth have been noted in in vivo and in vitro studies (Vertucci., 1984; Saini et al., 1990; Caliskan et al., 1995; Al-Quadah and Awawdeh; 2006; Weng et al., 2009; Al-Fouzan et al., 2012; Aminsobhani et al., 2013; Altunsoy et al., 2014; Lin et al., 2014; Zhao et al., 2014; Arslan et al., 2015; Zhengyan et al., 2016; da Silva et al., 2016; Martins et al., 2017; Verma et al., 2017; Mirhosseini et al., 2017; Saati et al., 2018; Martins et al., 2018; Martínez et al., 2018; Popovic et al., 2018; Shemesh et al., 2018; Razumova et al., 2018; Mashyakhy M., 2019; Valenti-Obino et al., 2019; Mashyakhy and Gambarini., 2019; Pan et al., 2019; Bourzgui and Akarslan., 2020; Ghabbani et al., 2020; Baxter et al., 2020) (Table 1), and in case reports (Hwang and Min., 2005; Al-Madi., 2020) (Table 2).

However, only a few of those studies were original research by Saini et al., 1990; Al-Fouzan et al., 2012; Mashyakhy M., 2019; Mashyakhy and Gambarini., 2019; Ghabbani et al., 2020, or case reports by Al-Nazhan., 1991; Alenazy et al., 2019; Al-Madi., 2020, conducted in Saudi Arabia. Here we summarize the published Saudi studies and investigate the number of canals and root morphology of maxillary and mandibular anterior teeth and making comparisons with the global data in relation to the original researches or case reports.

Search Methodology: All peer-reviewed original research articles or case reports for maxillary and/or mandibular central and lateral incisors from 1980 to 2020 addressing root or root canal morphology of permanent teeth were included in this review. A systematic literature review was carried out using the PubMed, Science Direct, Scopus, Evidence-Based Dentistry, and Journal of Evidence-Based Dental Practice databases. The search terms used were: "root canal morphology", "root morphology", "case report for anterior maxillary and mandibular teeth", and "Saudi Arabian population". All irrelevant or duplicate articles were excluded; the full texts of whole original researches or case reports were screened and saved in a single folder. In addition, all volumes or issues of the Saudi Dental Journal and Saudi Endodontic Journal were manually investigated for related topics. Lastly, the article reference lists were checked for further eligible articles.

Data Collections Original Researches: A total of 29 original research studies (18 mandibular teeth, seven maxillary teeth, four both teeth) were identified. Nineteen of the maxillary teeth studies were clinical studies that used CBCT, and three were laboratory studies using the clearing technique. Seven of the mandibular teeth studies used CBCT and a single study used radiograph, while the other three used the clearing technique. Three of the studies were original Saudi research involving mandibular teeth (Al-Fouzan et al., 2012; Mashyakhy., 2019; Ghabbani et al., 2020), were done in Al Madinah Al Munawara, Jazan, and Riyadh, respectively.

Also, two Saudi studies, done in Riyadh and Jazan (Saini et al., 1990; Mashyakhy and Gambarini., 2019), involved maxillary teeth. The author names, the year the study was conducted, country, sample size, type of tooth, genders, and anatomical features and finding are given

in Table 1. Also, significant differences were recorded when comparing genders, sides, types of teeth, bilateral symmetry, and techniques used. The anatomical features and finding in relation to Vertucci's classification were investigated, including the number of roots, number of root canals and their configurations, and other radiographical or anatomical findings.

Table 1. Summary of clinical and laboratory morphological studies of roots and canals number, and canals' configuration of mandibular and maxillary anterior teeth conducted on SA and worldwide countries

Researcher (S), Year/Country	Anatomical Features & %, general finding Tooth Type/ Vertucci Classification Type (N %) Gender I II III IV V VI VII Others Two canal	Sig ↔ Teeth, Gender & Bilateral symmetry, Male: Female %
	Mandibular teeth	
Ghabbani et al, 2020/KSA, Al-Madinah Al-Munawara[3]	Central 24.6% 0.00% 21.5% 1.2% 2.4% 1.0% Lateral 25.6% 0.00% 20.6% 1.2% 7.1% 1.6% Males 45.2% 0.00% 46.9% 0.00% 6%, 1.7% Female 64.8% 0.00% 29.4%, 0.9%, 3.3% 1.4%	SD ↔ Type Teeth & SD ↔ Genders Low Symmetry SD↔Saudi& Non-Saudi *Male 300/Females 106*
Mashyakhy M, 2019/ KSA, Jazan[4]	Central 73.7% 0.00% 26.3% 26.3% Lateral 69.2% 0.00% 29.8% 0.00% 1.0% 30.8% M/F 67.3%/79.4 0.0%/0.0% 32.7%/20.6% 2.7%/20/6%	SD ↔ Canal, Side, Type NSD- Gender Moderate Symmetry *M 48% Fe 52%*
Al-Fouzan et al, 2012/KSA, Riyadh[5]	Central 70% 0.00% 30% Lateral 70% 0.00% 30%	Very High symmetry
Baxter et al, 2020/ Germany[6]	Central 76.4% 22.3% 0.00% 0.7% 1.2% 22.6% Lateral 76.3% 21.4% 0.00% 0.00% 1.0% 24.3%	NSD ↔ Teeth, Age&SD ↔ Genders High Symmetry/ *M 116/Fe 186*
Mirhosseini et al, 2019/ Iran[7]	Central 76.1% 0.00% 15.8% 0.6% 7.6% 23.9% Lateral 65.0% 0.6% 15.7% 0.9%) 17.9% 35.0%	SD ↔ Tooth Type Low Symmetry
Pan et al, 2019/ Malaysia[19]	Central 94.9% 0.00% 1.0% 0.00% 4.2% Lateral 87.8% 0.00% 3.8% 0.3% 8.3%	NSD↔ Gender& Side Low Symmetry */M 43.3 /Fem56.7*
Valenti-Obino et al, 2019/Italy[8]	Central 55.0% 34.3% 9.3% 0.6% 0.8% 45% Lateral 57.0% 35.7% 6.9% 0.0% 0.4% 43%	NSD↔ Teeth Type High Symmetry
Razumova et al, 2018/ Russia[20]	Central 99.4% 0.00% 0.6% Lateral 99.2% 0.8% 0.0%	High Symmetry
Saati et al, 2018/Iran[9]	Central 54.5% 0.00% 34.2% 0.00% 11.3% Lateral 56.5% 0.00% 26.1% 0.00% 17.4%	NSD ↔Teeth Type NSD ↔ Gender/ High symmetry
Martins et al, 2018, China & Portugal[10]	Central 72.6% 2.40% 0.8% 0.00% 0.3% VII; 0.5% Lateral 70.1% 6.10% 23.1% 0.00% 0.2% VII; 0.3%	SD ↔Ethnics Moderate Symmetry
Martínez et al, 2018/ Belgium & Chile[11]	Central 60.50% 0.58% 32.18% 0.00% 4.02% VII; 0.58%, X; 1.15% Central 59.65% 0.58% 37.44% 0.00% 1.75% VII; 000% X; 0.58%	SD ↔ Ethnics Moderate Symmetry

Table 1. continue

Popovic et al, 2018/ Serbia[12]	Central 68.7% 7.2% 22.0% 0.00% 1.2% Lateral 72.0% 4.7% 22.0% 0.00% 1.2%	NSD↔ Tooth Type & SD ↔ Gender Moderate symmetry
Shemesh et al, 2018/ Israel[13]	Central 51.2%, 5.77% 39.15% 1.24% 0.62% 1.87% Lateral 56.96% 5.51% 35.83% 0.46% 0.00% 1.53%	NSD↔ Tooth Type & SD↔ Genders Moderate symmetry/ *M 653/F 855*
Verma et al, 2017/ India[14]	Central 68.3% 11.0% 15.3% 1.8% 3.8% M 15.2% Lateral 65.0% 13.3% 5.3% 3.0% 3.5% F 20.4% 1root &1canal 66.5% Man; 2canals 33.5%RC&L, 36.5%L	SD ↔ Side & SD ↔ Gender Moderate Symmetry& *103 M/97F*
Zhengyan et al., 2016/ China[15]	Central 96.3% 0.2% 2.7% 0.1% 0.75% 3.8% Lateral 89.4% 1.1% 7.7% 0.3% 0.70% IX; 0.3% 10.8%	SD ↔ Tooth Type& Side, Gender& Age Low Symmetry- *M 923 /Fee 802*
Arslan et al, 2015/ Turkey[16]	Central 51.9% 4.3% 41.6% 0.00% 0.5% 1.6% Lateral 37.2% 5.2% 55.2% 0.00% 1.7% 0.6%	SD ↔ Gender & Low Symmetry 54 Females &47 Males
Altunsoy et al, 2014/ Turkey[21]	Central 80.7% 0.6% 1.3% 4.2% 13.1% Lateral 76.7% 1.6% 1.4% 5.9% 14.4%	SD ↔ Gender & Moderate Symmetry- *410 Male /417 Female*
Lin et al, 2014/ China[30]	Central 89.1 % 2.4 % 6.2% 1.7 % 0.6 % Lateral 74.5 % 3.7 % 19.3% 2.1 % 0.4 Type III incisors most prevalent, followed types II, IV & V	SD↔ Tooth Type SD ↔ Gender High Symmetry *M 163 /Fe 190*
Zhao et al., 2014/ China[31]	All C& L single root/Central6.7%/Lateral7.4%/ III prevalent 2root canals 9.8% in 31-40 years in Cs& 21.5% (31-40 years) in L	SD↔ Tooth type & Age Groups Moderate Symmetry

Table 1. Continue

Aminsobhani et al, 2013/ Iran[32]	Central 72.7% 11.3% 4.7% 7.7% 3.6% 27.3% Lateral 70.6% 7.10% 3.7% 15.4% 3.2% 29.4%	NSD ↔ Gender High Symmetry/ *M 620/Fe 626*
AlOah&Awdeh, 2006/ Jordan[33]	73.8% 26.2% 8.7% had two separate apical foramina.	
Vertucci FJ, 1984/ USA[22]	Central 70% 5% 22% 0.00% 3% Lateral 75% 5% 18% 0.00% 2%	Moderate Symmetry
	Maxillary teeth	
Mashyakhy & Gambarini, 2019 / KSA, Jazan[17]	Central 100% Lateral 100%	NSD ↔ Gender Very-High Symmetry *M52/Fe 48*
Saini et al, 1990/ SA,Riyadh[18] (Shovel-shaped incisors)	C-Type I;0.90%, II; 3.73%, III; 3.25%, IV; 7.8%/ Type II C 4.48% L-Type I; 1.96%, II; 6.81%, III; 1.21%, IV:10% Type II L 11.11%	NS ↔ Genders Dens - invaginatus
Martins et al, 2018/ China & Portugal[10]	Central Asian 100% and White 100% Lateral Asian 100% and White 100%	SD ↔Ethnics
Pan et al, 2019/ Malaysia[19]	Central 94.9% 5.1% Lateral 87.85 12.3%	NSD ↔ Gender NSD ↔ Side High Symmetry
Razumova etal,2018/Russia[20]	Central 100% Lateral 100%	High Symmetry
Martins et al, 2017/ Portugal[35]	Central 100% Lateral 100%	Very High Symmetry
de Silva et al, 2016/ Brazil[36]	Central 98% 1.0% 0.00% 0.00% 1.0% Lateral 96% 3.5% 0.00% 0.00% 0.5%	Moderate Symmetry
Altunsoy et al, 2014/ Turkey[21]	Central 99.5% 0.00% 0.4% 0.00% 0.1% Male ↑ Lateral 99.7% 0.00% 0.00% 0.00% 0.3% Central 96.7% 1.3% 0.7% 0.00% 1.3% Female ↓ Lateral 98.3% 0.7% 0.00% 0.5% 0.5%	SD ↔ Gender Moderate Symmetry *Male 410 /Females 417*
Weng et al, 2009/ China[37]	Central 95.8% 4.2% 0.00% 0.00% 0.00% Lateral 91.4% 2.9% 1.40% 0.00% 4.3%	Moderate Symmetry
Caliskan et al, 1995/ Turkey[38]	Central 100% Lateral 78.05% 2.44% 14.63% 0.00% 4.88%	Moderate Symmetry
Vertucci FJ, 1984/ USA[22]	Central 100% Lateral 00%/24 lateral canal MAXC;1% cervical,6% medial,93% apical	Very High Symmetry

Few studies in the peer-reviewed literature have investigated the canal and root configuration of the maxillary arches (Table 1). A single Saudi study was carried-out by Mashyakhy and Gambarini., 2019 among a subpopulation in Jazan city, and they found that all maxillary central and lateral incisors were Vertucci's classification Type I. Another earlier study, published earlier, investigated shovel-shaped and dens invaginates in maxillary central incisors (Saini et al., 1990). Other international studies (Russian, Chinese, and Portuguese participants) reported the same percentages (100%) for their maxillary central and lateral incisors samples, and most of them were Vertucci's classification Type I (Martins et al., 2017; Razumova et al., 2018; Bourzgui and Akarslan., 2020). Other studies conducted in American, China, and Turkey (Vertucci., 1984; Caliskan et al., 1995; Weng et al., 2009 Altunsoy et al., 2014) reported Type I and Type III rates of 78.05% to 99.5%, respectively, for maxillary incisors. Vertucci's classification Type V was recorded in 1–4.88% of patients from China, Turkey, and Brazil (Weng et al., 2009; Altunsoy et al., 2014; da Silva et al., 2016).

Case Reports: Twenty-nine clinical case reports are listed in Table 2: 25 cases involving maxillary teeth and four cases including mandibular teeth. Among these, three cases were related to Saudi patients (Al-Nazhan S., 1991; Alenazy et al., 2019; Al-Madi EM., 2020). Most of the maxillary case reports were central incisors teeth ''21 of 25 in the maxilla''; nine cases were males and 14 females; and 14 cases were on the left side. Most canals were Vertucci's classification Type IV in both arches, and most of the teeth had two roots. Relatively few cases relating to the mandibular arch have been published. The following information's were gathered: the author(s) name, time of publication of the case report, place of documentations, gender, type and side of involved tooth/ teeth, number of canals, roots or canal configuration according to Vertucci's classification, and special finding associated with the treated case, if any. Figure 1 shows a radiograph of a treated case for male on the left lateral incisor maxillary tooth and other mandibular case for a female patient on the left central incisor tooth.

RESULTS AND DISCUSSION

Knowledge of tooth morphology is main basis for science of RCT. Today, root apex is not the only area in RCT science but the idea of three-dimensional RC filling implies that although working length and maintaining it is more important, access to all complications of canal inside is also crucial in order to RC filling (Castelucci., 2015; Bansal et al., 2018). Worldwide, the maxillary and mandibular central and lateral incisors are among the most likely teeth to require RCT (Castelucci., 2015; Filpo–Perez et al., 2015; Ahmed and Hashem., 2016; Martinsa et al., 2020; Baruwa et al., 2020).

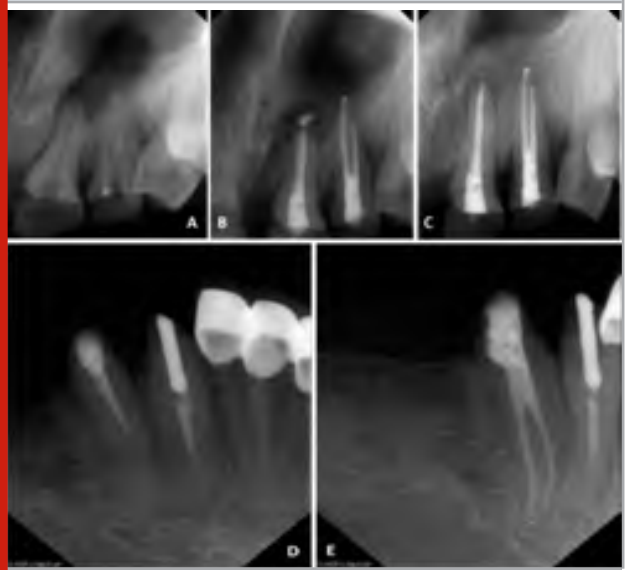
Table 2. Summary of previous case reports of Maxillary and Mandibular central and lateral incisors with variations as gender, tooth type, side, and canal morphology according to Vertucci's Classification

Author (s) & Year Publication	Country/ Gender	Vertucci's Classification and Tooth Type	Canal (s)	Root (s)	Special findings
		Maxillary Teeth			
Present Case	Figure 1 (A-C)/M	V/ Lateral, left	1	2	Non - Vital
Al-Madi et al, 2020[39]	Saudi Arabia/F	IV/ Central, left	2	2	Re-treatment
Al-Nazhan S, 1991[40]	Saudi Arabia/F	IV /Central, left	2	2	Enamel Hypoplasia
Buonvere & Buoivere, 2019[42]	Italy/F	VIII/ Central, left	1	3	Non-vital
Elbay et al, 2016[43]	Turkey/F	IV/ Central, right	2	2	Non-vital
		IV/ Lateral, right	2	2	
Sharma et al, 2014[44]	India/M	V/ Central, right	1	2	Crown dilaceration
Krishnamurti et al,2012[45]	India /F	Central, right	2	1	Root resorption
Kottoor & Murugesan, 2012[46]	India/M	Lateral, left	1	4	Non-vital
Nabavizadeh et al,2010[47]	Iran /M	IV/ Central, left	2	2	Non-vital
Gondim et al, 2009[48]	Brazil/M	Central, right	2	3	---------
Shokouhinejad et al, 2009[49]	Iran /F	Lateral, left	1	2	Non-vital
Rodrigues & Silva, 2009[50]	Brazil/F	IV/ Central, right	2	2	Non-vital
Sheikh-Nezami MM, 2007[51]	Iran /M	Central, right	1	3	Non-vital
Sponchiado et al, 2006[52]	Brazil /F	IV/ Central, left	2	2	---------
Lin et al, 2006[53]	China /F	IV/ Central, right	2	2	----------
Benenati FW, 2006[54]	--------	IV/ Central, left	2	2	Non-vital
Khojastehur & Khaya, 2005[55]	Iran/F	IV/ Central, left	2	2	Non-vital
Zaitoun &tMackie, 2004[56]]	U K /F	VIII/ Central, right	1	3	Non-vital
Genovese & Marsico,2003[57]	Italy /F	IV/ Central, right	2	2	Non-vital
Cimilli & Kartal, 2002[58]	Turkey/M	IV/ Central, left	2	2	Fusion of roots
Cabo-Vale & Gonz-Goez, 2001[59]	Spain/F	IV/ Central, right	2	2	Non-vital
Mangani & Ruddle, 1994[60]	Italy /F	Central, right	1	4	Dens invaginatus
Lambruschini & Camps, 1993[61]	France /F	IV/ Central, right	2	2	--------
Hososmi et al, 1989[62]	Japan/M	Central, right	2	3	Gemination
Mader & Konzelman, 1980[63]	U S A/M	IV/ Central, left	2	2	-----
Sinai & Lustbader, 1980[64]	U S A/--	IV/ Central, right	2	2	Incomplete apical formation
		Mandibular Teet			
Present Case	Figure 1 (D-E) /F	V/ Central, left	2	1	Re-treatment
Al Enazay et al., 2019[41]	Saudi Arabia /F	V/ Central, left	1	2	Re-treatment
		III/ Lateral, left	1	1	
		III/ Central, right	1	1	
		IV/ Lateral, right	2	1	
Hwang & Min, 2005[65]	South Korea	IV/ Central, R & L	2	2	Re-treatments
		IV/ Lateral, left	2	2	
Kabak & Abbott, 2007[66]	Belarusia/M	II/ Central, right	2	2	Non -vital
		II/ Lateral, R & L	21	22	
Guan et al., 2009[67]	China	IV Central, R & L	2	2	
		IV/ Lateral, R & L	2	2	----------

Usually, there is just one canal in the anteriors incisors (Vertucci., 1984; Altunsoy et al., 2014; Razumova et al., 2018; Mashyakhy and Gambarini., 2019; Pan et al., 2019). However, a second canal or other variations do occur (Al-Quadah and Awawdeh., 2006; Aminsobhani et al., 2013; Arslan et al., 2015; Zhengyan et al., 2016; Verma et al., 2017; Saati et al., 2018; Martins et al., 2018; Martínez et al., 2018; Popovic et al., 2018; Shemesh et al., 2018; Ghabbani et al., 2020). Anteriors incisors are the smallest human permanent teeth; incisors have complex roots and canals, especially mandibular incisors. Incisors can be single-rooted, have double roots or canals, a lateral

branch of a root canal, apical ramification, or apical furcation; this variability can complicate RCT (Hwang and Min., 2005; Kabak and Abbott., 2007; Guan et al., 2009; Elbay et al., 2016; Alenazy et al., 2019; Buonvivere & Buonvivere., 2019).

Figure 1: Maxillary left lateral incisor with preapical pathosis (A), teeth after RCT for lateral with two canals (B), follow-up after 18 months (c). Mandibular left lateral incisor with incomplete RCT (D), tooth after RCT with two root canals (E).



Here we review local and international studies and describe the numbers of canals and root morphology of maxillary and mandibular anterior teeth.Starting in the 20th century, the outside and inner structure of the maxillary and mandibular anterior teeth have been evaluated using in vivo and in vitro techniques. The in vivo techniques include clinical evaluation during RCT, retrospective assessment of patients' files, and radiographic analysis using conventional and advanced radiographic methods, such as CBCT. The in vitro techniques include root sectioning, canal staining, tooth clearing, microscopic and radiographic examinations using traditional or conventional x-rays, and 3-D techniques, such as micro-computed tomography (Neelakantan et al., 2010; Grover and Shetty., 2012; Filpo–Perez et al., 2015; Ahmad., 2015; Ahmed and Hashem., 2016; Przesmycka and Tomczyk., 2016; Martins et al., 2019; Martinsa et al., 2020).

For mandibular central incisors, multiple studies have been published, conducted in various countries. The percentages of Vertucci's classification Type I for central incisors were above 70% and exceeded 90% in some local Al-Fouzan et al., 2012; Mashyakhy., 2019;, and international (Al-Quadah and Awawdeh., 2006; Altunsoy et al., 2014; Mirhosseini et al., 2017; Baxter et al., 2020) studies. Ghabbani et al 2020, ''Saudi Arabia'' reported a lower percentage. Similar percentages were documented in other countries, such as Italy, Iran, Israel, and Turkey (Arslan et al., 2015; Saati et al., 2018; Shemesh et al., 2018; Valenti-Obino et al., 2019).

Also, the rate of Type I Vertucci's classification among the mandibular lateral incisors of Saudis has been reported to be as high as 70% (Al-Fouzan et al., 2012; Mashyakhy., 2019),and similar rates have been reported in the US, Turkey, Iran, Portugal, and Germany, (Vertucci., 1984; Altunsoy et al., 2014; Mirhosseini et al., 2017; Martins et al., 2018; Baxter et al., 2020). The frequency of Vertucci's Type III in mandibular lateral incisors was recorded as 20–30% in Saudi studies by Al-Fouzan et al., 2012; Mashyakhy., 2019; Ghabbani et al., 2020; and similar findings were reported by (Satti et al., 2018) in Iran, (Martins et al., 2018) among patients from China and Portugal, and Turkish patients by (Arslan et al., 2015), and less than 20% amongst patients from Iran, Belgium & Chile, and Germany (Mirhosseini et al., 2017; Martínez et al., 2018; Baxter et al., 2020).

Local studies in Saudi Arabia have reported that around 30% of mandibular teeth had two canals (Al-Fouzan et al., 2012; Mashyakhy., 2019; Ghabbani et al., 2020). This is well supported by Ahmed et al., 2015. who reported that the two-canal configuration is the most common accessory anatomical variation in single-rooted mandibular anteriors (Ahmed and Hashem., 2016). This is in line with other studies conducted in, Iran, India, Serbia, and Germany (Mirhosseini et al., 2017; Verma et al., 2017; Popovic et al., 2018; Baxter et al., 2020) but a higher percentage was detected in Turkey, Israel, and Italy (Arslan et al., 2015; Shemesh et al., 2018; Valenti-Obino et al., 2019). Also, two canals were more common amongst females than males (Verma et al., 2017), but this trend was reversed in a Turkish population (Altunsoy et al., 2014). Finally, mandibular lateral incisors with two canals were more than central incisors among a Chinese population (Zhao et al., 2014). Vertucci's classification Type IV was the least common in all studies, and Type V was present in small percentages within the screened patients in most of the studies (Table 1).

The root canal morphology can change over time. Changes due to normal physiological aging usually occur because of secondary dentine deposition (Johnstone and Parashos., 2015). A recent study has reported high variability in root canal morphology of mandibular anterior incisors. Vertucci's classification Type VII was detected in a local study conducted in Al Madinah Al Munawara (Ghabbani et al., 2020). Also, a similar canal Type was reported in those studies that included participants from Turkey, China, Portugal, Belgium, Chile, and Israel (Arslan et al., 2015, Martins et al., 2018; Martínez et al., 2018; Shemesh et al., 2018;). Other variations were shown in the form of Types IX and X in China and Belgium (0.58–1.15%, respectively), and in China alone (10.8%) (Weng et al., 2009; Bourzgui and Akarslan., 2020).

Compared to the mandibular arch, relatively few studies have addressed maxillary anteriors (Vertucci., 1984;

Caliskan et al., 1995; Weng et al., 2009; Altunsoy et al., 2014; da Silva et al., 2016; Martins et al., 2017; Razumova et al., 2018; Mashyakhy and Gambarini., 2019; Pan et al., 2019; Bourzgui and Akarslan., 2020). Both central and lateral maxillary teeth typically start with a single canal and end in a single root. Rates of up to 100% Vertucci's Classification Type I have been reported, including in a single local Saudi study (Mashyakhy and Gambarini., 2019), and studies carried out in Russia, America, China, Portugal, and Turkey (Vertucci., 1984; Caliskan et al., 1995; Martins et al., 2017; Razumova et al., 2018; Bourzgui and Akarslan., 2020), but lower rates have been reported amongst central maxillary teeth in studies conducted in China, Brazil, and Malaysia (Weng et al., 2009; da Silva et al., 2016; Pan et al., 2019).

On the other hand, a rate of 78–91%; Weng et al., 2009 was recorded for maxillary lateral incisors in Turkey, China, and Malaysia (Caliskan et al., 1995; Pan et al., 2019;). Few studies reported a moderate percentage of two canals in maxillary anteriors or incisors, with lower rates than in mandibular teeth. Vertucci's classification Type III and IV were relatively rare. In a study conducted by Altunosy et al., 2014 among a Turkish population, the authors reported that two canals were more common in males than females; another study reported significant differences in canal number and configuration when comparing populations from China and Portugal (Bourzgui and Akarslan., 2020).

In this review, we conducted a gender comparison in relation to the number of canals, the number of root canals, and root canal configurations (according to Vertucci's classification). Both studies among Jazanian publications showed no significant differences between gender (Mashyakhy., 2019; Mashyakhy and Gambarini., 2019), while a study conducted in Al Madinah Al Munawara by Ghabbani et al., 2020 showed a significant difference between genders. This could be explained by the uniform sample of the population in Jazan, and mixed populations in the study conducted by Ghabbani et al., 2020 and both studies conducted in Turkey by Altunsoy et al., 2014 and Arslan et al., 2015, while no significant differences were recorded in Malaysia in both arches Pan et al., 2019, or both Iranian studies by Aminsobhani et al., 2013; Saati et al., 2018).

The bilateral symmetry between sides in relation to the type of teeth and canals configurations as well as root numbers, Al-Fouzan et al., 2012 reported a high or typical symmetry between the extracted mandibular teeth in relation to the number of canals and canal configurations. This is consistent with studies conducted in Europe 'Italy and Germany'' (Valenti-Obino et al., 2019; Baxter et al., 2020), Asia ''China and Iran'' (Lin et al., 2014; Saati et al., 2018), and the US (Vertucci., 1984), which have also reported high symmetry, which might be related to the racial type and uniform sample types. Mashyakhy M., 2019, Mashyakhy and Gambrini., 2019 reported moderate bilateral asymmetries in central and lateral maxillary and mandibular incisors in relation to some canals and canals configurations. This finding is similar to those from other countries, such as studies conducted in India, China, Portugal, Belgium, Serbia, and Israel (Verma et al., 2017; Martins et al., 2018; Martínez et al., 2018; Popovic et al., 2018; Shemesh et al., 2018).

In Al Madinah Al Munawara, Ghabbani et al., 2020 reported low symmetry among the participants. This is likely because Muslims from all around the world visit this city and many of them stayed for a long time. Also, his sample was a mix of Saudis and non-Saudis, and a significant difference was recorded between these groups. International studies conducted in Iran, China, Turkey, and Malaysia, including mixed races of (Malawians, Chinese, and Indianans) addressing maxillary incisors have reported a low percentage of bilateral symmetry (Arslan et al., 2015; Zhengyan et al., 2016; Mirhosseini et al., 2017; Pan et al., 2019).

A high bilateral symmetry for maxillary teeth was recorded by Mashyakhy and Gambarini., 2019 among Saudis subpopulations, which was consistent with international findings among subjects from America, Portugal, Russia, and Malaysia by Vertucc., 1984; Martins et al., 2017, Razumova et al., 2018; Pan et al., 2019, while moderate bilateral symmetry was detected among samples involving the mandibular incisors among Chinese, Turkish, and Brazilin participants (Weng et al., 2009; Altunsoy et al., 2014; da Silva et al., 2016). Overall, the Saudi studies documented significant differences between central and lateral teeth regarding Vertucci's classification of the canal and root canal type, or number and configurations. Similar findings reporting significant differences between mandibular teeth type were found in Germany and India (Verma et al., 2017; Baxter et al., 2020), while the opposite was documented in Turkey, Serbia, Malaysia, and Israel, (Altunsoy et al., 2014; Popovic et al., 2018; Pan et al., 2019; Valenti-Obino et al., 2019;), and with mixed results in Iran and China (Aminsobhani et al., 2013; Lin et al., 2014;).

Most case reports of maxillary and mandibular anterior teeth described a different pulp morphology or number of canals, as well as roots (Sharma et al., 2016 - Baruwa et al., 2020). Some Saudis case reports have mentioned dissimilarities in canal anatomy in relation to gender, arch, and tooth type ''Table 2 and Figure 1'' There are 25 case reports for maxillary teeth (Table 2) (Al-Nazhan., 1991; Alenazy et al., 2019; Al-Madi., 2020) and (Sinal & Lustbader., 1984; Buonvivere and Buonvivere., 2019). Two local cases were reported, by Al-Nazhan., 1991and Al-Madi et al., 2020. The first case was for a female patient with maxillary left central incisor, while. the second reported a female patient with re-treatment for a left maxillary central incisor tooth. The canal morphology for both cases was Vertucci's classification Type IV, with two canals and two roots. The second case was associated with enamel hypoplasia, while the first was discovered during re-treatment of the teeth.

Figure 1 (A–C) shows a male patient with a non-vital left central maxillary incisor with Vertucci's classification Type V. In the maxillary teeth, there were more cases

among females than males (15 vs. 10 cases), most were central incisors (22), and left-sided (15 vs. 10 right-sided), and all of the published reports involved a single tooth only. Both published cases by Al-Nazhan., 1991; Al-Madi et al., 2020 and were consistent with the international case reports (Mader and Konzelman., 1980; Sinal and Lustbader., 1984; Lambruschini and Camps., 1993; Mangani and Ruddle., 1994; Cabo-Valle and Gonzalez-Gonzalez., 2001; Cimilli and Kartal., 2002; Genovese and Marsico., 2003; Khojastehpour and Khayat., 2005; Sponchiado et al., 2006; Lin et al., 2006; Benenati., 2006; Krishnamurti et al., 2012; Elbay et al., 2016) in relative to the tooth type, side, gender, and Vertucci's classification (17 cases out of 25 with Type IV), as well as canal configuration and root numbers.

Central maxillary incisures have a tendency for a more abnormal canal and root configurations due to the presence of dens invaginatus and shovel-shaped incisors (Saini et al., 1990; Mangani and Ruddle., 1994; Ahmed and Hashem., 2016), enamel hypoplasia (Al-Nazhan., 1991), and fusion roots (Cimilli and Kartal., 2002). Saini et al., 1990, measured the rate of shovel-shaped incisors among Saudis and detected different types among central incisors and different classification of dens invaginatus among the participants. Baruwa et al., 2020 reported a failure rate greater than 7% among maxillary central incisors due to either missed canals associated with a periapical lesion in 116 canals or with 119 canals without periapical lesions among 1693 screened teeth.

We identified relatively few case reports involving mandibular teeth. However, there were many research studies addressing central and lateral incisor mandibular teeth (Table 2). Al Enazay et al., 2019 reported the case of a Saudi female patient with unusual canal configuration and anatomy of the roots in her mandibular central and lateral incisors during re-treatments of the same teeth. This case showed the four mandibular teeth having different Vertucci's classification: Type V for the center-left, Type IV for lateral right, while lateral left and central right were with Vertucci's classification Type III (Alenazy et al., 2019). Internationally, Guan et al. (China) and Hwang and Min (South Korea) (Hwang and Min., 2005; Guan et al., 2009) reported cases with Vertucci's classification Type IV for all mandibular central and lateral incisors. In these studies, Type IV is relatively more common that in our cases, while (Kabak and Abbotto., 2007) from Belarusians had reported Vertucci's classification Type V for the same teeth.

Figure 1 (D and E) shows the mandibular left central incisor of a female patient in with Vertucci's classification Type V. These differences in the types might be explained in multiple ways, such as differences in ethnicities, age, and the genetic factors of the patients. Ahmed et al. noted that the prevalence of accessory root/root canal dissimilarities in the mandibular anterior teeth is greater than maxillary complements (Grover et al., 2012; Johnstone and Parashos,. 2015; Ahmed and Hashem., 2016). Root canal configuration types 2-2 and 1-2 are the most common accessory anatomical variations in single-rooted mandibular anteriors (Ahmed and Hashem., 2016). Due to the wide morphological variance of the root and root canal system in human anterior teeth, dental general practitioners and specialists should be aware of such anatomical variations, thereby decreasing the risk of failure because of inadequate debridement of inaccessible or undetected parts of the RCS. Recently developed diagnostic devices, the latest endodontic (and periodontal) techniques, and improvements in RCT biomaterials and machines are likely to improve RCT outcomes for patients (Ahmed and Hashem., 2016).

CONCLUSION

From this review the concluding remarks can be drown; The Saudi data and data gathered internationally in relation to canal number, root canal configuration was largely consistent. Type I Vertucci's classification of canal configuration is the most common type in both arches, followed by Type III. Variations are frequent in the mandibular teeth. Other types of Vertucci's classification were present in all populations, but at lower rates. The bilateral symmetry of canal and root numbers and configuration vary across populations and genders. All reported cases describing maxillary teeth included only a single tooth, while most of the cases involving mandibular anterior teeth described an unusual canal configuration or root canal number. Dentists should be familiar with the variations in canal numbers and configuration. Also, dentists should know how to use at least read the output of new technologies for visualizing root canal systems.

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Evaluation of Phytochemical, Antioxidant and Reducing Activity in Whole Plant Extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

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ABSTRACT

In the present study, phytochemical screening, antioxidant activity, polyphenolic activity and reducing power of *Andrographis paniculata* plant prepared in different solvents (methanolic, ethanolic and double distilled water) was assessed by different protocols.2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, Hydrogen peroxide (H_2O_2) radical scavenging activity, Polyphenolic contents and reducing activity of the plant was evaluted by modified method. Phytochemical screening of plant showed the presence of carbohydrate, cardiac glycosides, amino acids, flavonoids, alkaloids, phenols, saponins, steroids and tannins. In DPPH free radical and H_2O_2 radical scavenging activity , methanolic extract of plant were most potent in activity with 50% inhibition at 333.34 µg/ml. and 398.12 µg/ml concentration respectively. Total phenolic (309 ± 0.81 mg/g of gallic acid equivalent) and flavonoid content (82.125 ± 0.85 mg/g of rutin equivalent) were maximum in the methanolic extract of plant. High reducing capacity of plant was observed in case of methanolic extract. A significant positive correlation was found between antioxidant activity and polyphenolic content (total phenols and total flavonoids). Moreover, a significant correlation was found between antioxidant activities and reducing potential of plant extract, depicting that reducers are important contributors to antioxidant. The study shows whole plant extract of *A. paniculata* as an important natural source of antioxidants and phytochemicals.

KEY WORDS: ANDROGRAPHIS PANICULATA, WHOLE PLANT EXTRACT, ANTIOXIDANT ACTIVITY, POLYPHENOLIC CONTENT, DPPH FREE RADICAL ACTIVITY, H_2O_2 SCAVENGING ACTIVITY, REDUCING POWER.

INTRODUCTION

Medicinal plant is the future of phytomedicines (plant-derived drugs) and serves as a rich source of food, fibres and drugs. They have been used in folk medicine since ancient times for the prevention and treatment of the numerous diseases as they express a vast array of biological activities. Presently, research is focusing attention on medicinal plants as it is considered as the most sustainable alternative source of antioxidants to supplement the endogenous oxidative stress defense system in humans (Rahman et al., 2012 Banji et al 2028, Engwa 2018, Zwolan et al 2020).

Antioxidants obtained from the plants either in the form of crude extracts or their derived products is very effective to inhibit the destructive processes caused by oxidative stress (Zengin et al., 2011). Oxidative stress generates free radicals in form of reactive oxygen species (ROS) in the human body through aerobic respiration, ionizing radiation and pollution may increase the risk of chronic and degenerative diseases such as cancer, cardiovascular

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diseases, ageing and atherosclerosis (Valko et al., 2007). The human body generates antioxidant enzymes to neutralize free radicals (Rimbach et al., 2005), a diet rich in edible antioxidants is recommended to assist the human body to protect itself from food borne free radicals. Plants phytochemicals have shown to possess antioxidant properties capable of scavenging free radicals, preventing cellular damages and related diseases via several mechanisms. Hydrogen peroxide (H_2O_2), superoxide ion (O2–) and hydroxide radical (OH–) are considered as most common ROS. Antioxidants are the molecules which stabilize or deactivate free radicals, before they hit targets in living human cells (Nunes et al., 2012 Banji et al 2028, Engwa 2018, Zwolan et al 2020).

Plants contain a wide variety of free radical scavenging molecules, such as anthocyanins, carotenoids, flavonoids, glutathione, vitamins and endogenous metabolites (Zheng and Wang, 2001).The concentration of the phenolic compounds like phenolic acids, flavonoids, anthocyanins and tannins etc. may be related to the antioxidant activity of medicinal plants (Djeridane et al., 2006). Natural antioxidants have gained interest in pharmaceutical research as an alternative for substitution of synthetic substances showing antioxidant activity (Huang et al., 2005). It is mainly due to the fact that natural antioxidants are cost effective, easily available, non-toxic, eco-friendly and sometimes more efficient than synthetic ones. Continuous efforts are required to characterize plants phytochemicals for their antioxidant potentials and mode of action for various therapeutic uses against oxidative stress-related diseases, (Zwolan et al 2020).

Andrographis paniculata (Burm. f.) Wall. ex Nees of Acanthaceae family is commonly known as Kalmegh/ King of Bitter. The plant is gregarious and grows abundantly in moist, shady waste area and dry forests. It is extensively cultivated in southern Asia, some parts of Europe and China. Traditionally it is used for treating common cold, bronchitis, diarrhoea, fever, hypertension, liver disease and sinusitis (Gabrielian et al., 2002) band snake bite (Samy et al., 2008; Premchandran et al., 2011). Major constituents of *A. paniculata* are diterpenoids i.e. andrographolide, 14-deoxy-11,12-didehydroandrographolide, 14-deoxyandrographolide (Pholphana et al., 2013), neoandrographolide, flavonoids and polyphenols reported to possess the most potent hypotensive and vasorelaxing effect. The plant has been reported to exhibit multifarious pharmacological and biological properties (Niranjan et al., 2010) like antibacterial (Mishra et al., 2009), anticancer (Lim et al., 2012), antidiabetic (Akhtar et al., 2006), antifungal (Sule et al., 2012), anti-inflammatory (Chandrasekaran et al., 2010), anti-HIV (Nanduri et al., 2004) and antihepatotoxic (Nagalekshmi et al., 2011).

The plant showed potential therapeutic action in curing liver disorders, common cough and colds in humans (Geethangili et al., 2008). The present study was therefore performed to study the antioxidant and polyphenols of whole plant extract of *A. paniculata* in three different solvents, which may prove to be beneficial against free radical generated disorders. Reducing potential of the plant was evaluated for the first time in methanolic, ethanolic and aqueous extracts derived from the plant.

MATERIAL AND METHODS

Ascorbic acid, 2,2-Diphenyl,1-picryl hydrazyl (DPPH), gallic acid, rutin, trichloroacetic acid (TCA), potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, aluminium chloride ($AlCl_3$), rutin, sodium potassium tartarate (Na-K tartarate), sodium carbonate (Na_2CO_3) were purchased from Hi-Media Ltd and solvent ethanol and methanol used were of analytical grade and purchased from Merck (Darmstadt, Germany) *A. paniculata* plant was collected from the campus of Banaras Hindu University, Varanasi. The plant was washed under running tap water to remove the soil and dust particles. The plant was authenticated at Botanical Survey of India (BSI), Allahabad. Collection number BHU-173 and voucher number-91924 was given by BSI to plant flora. Whole plant consisting of (root, stem, leaf, seed, flower) was shade dried for one week and kept in an oven at 40-45°C for 24 h, and then grinded in an electrical grinder to make coarse powder. Extraction was done from 20 g of plant powder in 200 ml of solvent by using a soxhlet apparatus for 12 h. Methanol, ethanol and double distilled water were used as extraction solvents for extraction purpose. Extracts were then filtered and dried at 40°C in a rotary evaporator. Extracts were stored at 4°C till use. Percentage yield {PY, expressed in (w/w)} of crude plant extract was calculated by given formula:

$$PY = \frac{\text{Wt of crude extract recovered}}{\text{Wt of powder used}}$$

One gram of each extract was dissolved in 10 ml of respective extraction solvents to obtain a stock solution of concentration 100 mg/ml. Test plant samples were diluted in various concentrations according to the experiments.Phytochemical testing of the plant for various solvent extract was carried using a standard protocol (Harborne, 1973; Sofowora, 1993).

Antioxidant assay through DPPH: The free radical scavenging activity of the extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method given by McCune and Johns, (2008) with some modifications. One ml sample of various concentrations (100-600 µg/ml) of plant extract (PE) was added to 3 ml methanolic solution of DPPH (0.004%) and shaken vigorously. The mixtures were incubated in the dark for 15 min at room temperature. Ascorbic acid was used as standard and methanol served as blank. The solution without sample was served as control. The absorbance of the samples was recorded at 517 nm by using a spectrophotometer (UV1, Thermo Scientific, US). The experiment was expressed as the percent inhibition of free radicals by the sample and was calculated using the following equation:

$\text{DPPH activity}(\%) = \frac{(C-S)}{(C)} \times 100$ (C =Absorbance of control, S = Absorbance of sample).

Hydrogen Peroxide (H_2O_2) Scavenging Assay: The radical scavenging activity of methanolic, ethanolic and aqueous extracts of the plants to scavenge hydrogen peroxide (H_2O_2) was evaluated by the method of Ruch et al., (1989) with slight modifications. One ml sample of various concentrations (100-600 µg/ml) of plant extract (PE) were added to 2 ml of H_2O_2 (40 mM) prepared in (50 mM, pH-7.4) phosphate buffer. The test samples were incubated for 10 min at room temperature. The absorbance was measured at 230 nm (Thermo Scientific UV 1). Phosphate buffer without H_2O_2 was used for blank and hydrogen peroxide solution without extract served as control. Ascorbic acid was used as a standard. Hydrogen peroxide scavenging activity was calculated by following formula:

$$\text{Hydrogen peroxide scavenging activity}\ (\%) = \frac{(C-T)}{(C)} \times 100$$

Where, C = absorbance of control, T= absorbance of test sample

Total phenolic content (TPC) was measured by Folin-Ciocalteu assay (McDonald et al., 2001). In brief, 0.5 ml Folin reagent (1:10 diluted with DDW) was added to 0.5 ml (200 µg ml-1) PE and finally 4 ml (1M) aqueous sodium carbonate (Na_2CO_3) was added to this reaction mixture and incubated for 15 min at room temperature. Absorbance was recorded at 650 nm. Gallic acid was prepared in methanol and DDW (1:1) and used as standard. Total phenolic content was expressed in terms of gallic acid equivalent (GAE, mg/g of dry mass), which is a common reference compound. Total flavonoid content (TFC) was determined using the method of aluminium chloride ($AlCl_3$) (Chang et al., 2002). The plant extract (1 ml, different concentration) prepared with different solvent (methanol, ethanol and water) was taken in which 100 µl $AlCl_3$ (10% w/v), 100 µl Na-K tartrate and 2.8 ml distilled water were added and kept for 30 min. Finally, the reaction mixture was diluted to 10 ml with double distilled water and the absorbance was measured at 415 nm. The results were expressed as mg rutin (RE)/g plant material.

Reducing potential capacity of methanolic, ethanolic and aqueous plant extract was estimated by the method of Athukorala et al., (2006) with some modifications. In brief, 1ml of PE (50-300 µg/ml) prepared in different solvents were mixed with 2.5 ml of phosphate buffer solution (PBS, 0.2 M, pH- 6.6) and 2.5 ml potassium ferricyanide (30mM). The above reaction mixture was incubated at 50°C for 20 min. After that, 2.5 ml trichloro acetic acid (TCA, 0.6M) was added to the mixture to stop the reaction and centrifuged at 3000 rpm for 10 min. Then, 2.5 ml of supernatant was taken out and mixed with 2.5 ml double distilled water and 0.5 ml ferric chloride ($FeCl_3$) solution. Absorbance was recorded at 700 nm. Ascorbic acid was used as standard.

Statistical analysis: All the above experiments were performed in quadriplate (n=4) and repeated thrice (x=3). Data were analyzed as mean ± SE by applying one way analysis of variance (ANOVA). Tukey's multiple range tests were used for separation of means when ANOVA was significant ($p< 0.001$) (SPSS 16.0; Chicago, IL, USA). IC_{50} was calculated through linear regression analysis. The graphs were drawn in sigma plot 11.0.

RESULTS AND DISCUSSION

Percentage yield of A. paniculata extract was found maximum (22%) in aqueous followed by methanol (18.4%) and ethanol (17.6%) was obtained. The percentage yield of extract differed in various extraction solvents and this may be due to various degrees of solubility of plant materials depending on polarity of solvents. A similar trend was seen in leaves extract of *A. paniculata* (Banji et al., 2018). Our results highlight that methanolic and ethanolic extracts whole plant were enriched in phytochemicals like alkaloids, amino acids, carbohydrate, flavonoids, phenols, saponins, steroids and tannins while aqueous extract shows presence of alkaloids and amino acids only (Table 1). It may be due to poor solubility of these phytochemicals in the aqueous extract.

Table 1. Phytochemical screening of plant in different solvents

Phytochemicals	Test performed	Methanolic extract	Ethanolic extract	Aqueous extract
Carbohydrate	Fehling test	+	+	-
Phenols	Ferric chloride test	+	+	-
Flavonoids	Ammonia test	+	+	-
Alkaloids	Wagner's test	+	+	+
Steroids	Salkowski test	+	+	-
Tannins	Lead acetate test	+	+	-
Saponins	Frothing test	+	+	-
Glycosides	Nitroprusside test	+	-	-
Amino acids	Ninhydrin test	+	+	+

Note: + = Presence; - = Absence of phytochemicals

In the present study, the free radical scavenging ability of the crude methanolic, ethanolic and water extracts were determined through the degree of discoloration of the methanol solution of DPPH (Table 2). In *A. paniculata*, methanolic extract showed higher scavenging activity (IC_{50} = 398.31 µg/ml) than ethanolic (IC_{50} = 404 µg/ml) and aqueous extracts (IC_{50} = 483.29 µg/ml). The present study reveals that the best antioxidant activity in terms of DPPH scavenging strength was displayed by methanol extract followed by ethanol and aqueous extract. The higher antioxidative capacity of methanolic extract followed by ethanolic extract may be explained via the higher content of biologically active substances, such as e.g. polyphenol.

Ethanolic extract was characterized by higher free radical antioxidant activity than water extract in *Argyreia pierreana*, *Matelea denticulata* (Gudise et al., 2019) and *Nigella sativa* (Zwolan et al., 2020). The antioxidant activity of the extract is first estimated based on their capacity to trap free radical DPPH. In the presence of an active free radical scavenger, the absorption vanishes and the resulting discoloration from deep violet to light yellow. The solution fades colour with increase in concentration of antioxidant as electrons are taken up by DPPH radical from the antioxidant (Calliste et al., 2001) of the extract. Ascorbic acid was used as a standard antioxidant as used as a standard to determine the IC50 value of the extract in other plants (Sreekala et al., 2013).

Hydrogen peroxide (H_2O_2) scavenging activity of *A. paniculata* plant was observed higher in methanolic (IC_{50}= 377.074 µg/ml) followed by ethanolic (IC_{50}= 379.06 µg/ml) extract and aqueous extract (IC_{50}= 467.65 µg/ml) (Table-3). H_2O_2 scavenging activity relies upon the phenolic content of the plant extract by donating electrons to H_2O_2, thereby neutralizing it into water. The study suggests that aqueous extract will be required in relatively high concentration to show its effectivenss. The ethanolic extract of the *Aesculus hippocastanum* was capable of scavenging H_2O_2 in a dose dependent manner (Geetha et al., 2013). H_2O_2 radical scavenging activity was also reported from different extracts of *E. prostrata* (Sinha and Raghuwanshi, 2016a).

Table 2. Antioxidant activity of *A. paniculata* by DPPH free radical scavenging method in different solvents

Concentration (µg/ml)	Percentage inhibition (Mean±SE)			
	Methanolic	Ethanolic	Aqueous	Ascorbic Acid
100	23.89±0.68f	25.77±0.60f	17.07±0.34f	25.12±0.29f
200	30.52±0.63e	35.01±0.23e	24.71±1.9e	39.34±0.20e
300	43.09±0.68d	42.88±0.18d	34.10±0.59d	56.25±0.22d
400	49.92±0.82c	51.58±0.53c	43.69±0.72c	65.15±0.14c
500	59.11±1.04b	58.84±0.12b	51.94±0.51b	86.47±0.38b
600	63.81± 0.49a	63.65±0.19a	60.12±0.80a	95.22±0.32a
IC_{50}	398.31	404.00	483.29	271.47

Data represented as mean ±SE (n=4). One way ANOVA followed by Tukey's test. All data are significant at p<0.001; a,b,c,d,e,f = different letter shows significant difference between means.

Total phenolic content was reported as mg/g of GAE in reference to standard curve (y=0.001x+0.05, R2=0.997). In A. paniculata plant, maximum TPC (309.00±0.816 mg/g) was found in methanolic extract followed by ethanolic (290.5±1.29 mg/g) and aqueous extracts (189.25±0.957 mg/g) respectively. Total flavonoid content was calculated by standard curve (y=0.0008x+0.198, R2=0.994) and reported as mg/g of RE. A. paniculata plant showed maximum TFC (82.125±0.853 mg/g) in methanolic extract followed by ethanolic (61.375±1.10 mg/g) and aqueous extracts (37.80±0.731 mg/g) (Table 4). Methanol extract of *A. paniculata* shows important antioxidant activity because it contains phenols and flavonoids (Kurzawa et al., 2014). Similar, higher phenolic content in organic solvent has also been reported (Zaman et al., 2011).

Presence of active metabolites like phenol and flavonoid contents in plant extract depend on solvent used (Sulaiman et al., 2011). Phenolic compounds present in plant contain an aromatic ring bearing one or more hydroxyl groups. Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts in form as aglycone and glycosides. It has two benzene rings separated by a propane unit. Their ideal structural chemistry nature helps them to scavenge injurious free radicals such as super oxide and hydroxyl radicals (Younes and Siegers, (1981). Therefore, acting as antioxidants for their scavenging activity (Das and Pereira, 1990) or chelating process, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions (Clavin et al., 2007) and giving protection against cardiovascular disease, certain forms of cancer and age-

related degeneration of cell components. Flavonoids might show higher antioxidant activity in organic solvent due to structure and substitution pattern of hydroxyl group.

The reducing power of the extracts (methanolic, ethanolic and aqueous) of A. paniculata (Fig. 1) plant increased in a concentration dependent manner from lower to higher concentrations. Similar results reported by (Abdallah et al., 2016) in which the reducing power of *Ziziphus mauritiana* extract increased with the increase of their concentrations. Maximum reducing power was observed in the methanolic extract the plant. In reducing potential assay, after the addition of the extract, the yellow colour of the test solution changes from yellowish green to blue. The colour change of sample solution indicates the reducing power of extract of plants. High absorbance shows high reduction potential of the plant. These reducers show their antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Thus, it is concluded that both polyphenolic compounds and reducers present in the extracts are major determinants of antioxidant capacity of extracts.

Table 3. Antioxidant activity of *A.paniculata* by H_2O_2 radical scavenging in different solvents

Concentration (µg/ml)	Percentage inhibition (Mean±SE)			
	Methanolic	Ethanolic	Aqueous	Ascorbic Acid
100	21.07±0.79f	27.66±0.50f	20.57±0.70f	25.86±0.38f
200	31.68±0.63e	35.63±0.62e	27.88±0.62e	36.80±0.30e
300	43.17±0.56d	43.68±0.60d	36.00±0.34d	48.33±0.31d
400	51.81±0.83c	51.80±0.38c	44.62±0.49c	57.07±0.29c
500	56.69±0.50b	55.67±1.98b	54.75±0.85b	61.87±0.39b
600	62.87±0.78a	63.01±0.49a	59.62±0.87a	74.16±0.38a
IC_{50}	377.07	379.06	467.65	342.56

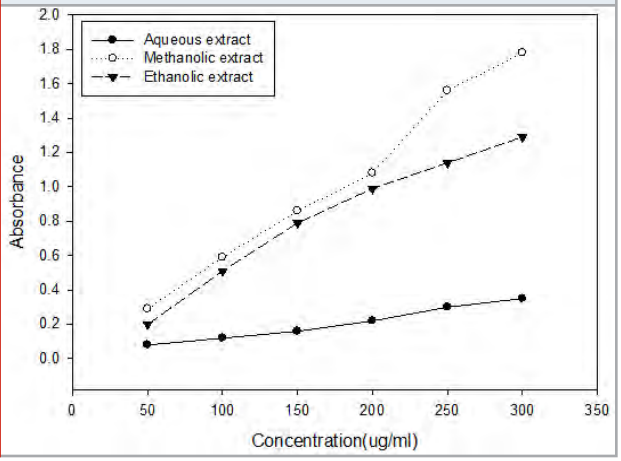
Data represented as mean ±SE (n=4). One way ANOVA followed by Tukey's test. All data is significant at p <0.001. a,b,c,d,e,f = different letters shows significant difference between means.

Table 4. Total phenolic and flavonoid content of *A. paniculata* in different solvents

Total polyphenolic content (A. paniculata)		
Plant extract	TPC (mg/g GAE)	TFC (mg/g RE)
Methanol	309 ± 0.816a	82.125±0.853a
Ethanol	290.5±1.290b	61.375±1.108b
Aqueous	189.25±0.957c	37.805±0.731c

Data represented as Mean ±SE (n=4); One way ANOVA followed by Tukey's test. All data is significant at p <0.001; a,b,c letters shows significant difference between means.

Figure 1: Reducing potential of *A. paniculata* plant extracts in different solvents



Correlation between total antioxidant activity and reducing power was obtained through linear regression analysis. A significant correlation was found between total antioxidant activities and reducing potential in *A. paniculata* extract (Fig. 2). In, *A. paniculata*, correlation coefficient (R2) between antioxidant activity and reduction potential was (R^2 =0.989) for methanolic, (R^2 =0.992) for ethanolic and (R^2 =0.992) for aqueous extract. Similar studies are seen in *E. prostrata* (Sinha and Raghuwanshi, 2016a) and *Ocimum americanum* (Jaiswal et al., 2019). In our result, there is significant positive correlation between antioxidant activity and reducing power of the plant. Koleva et al., (2002) also reported positive correlation between antioxidant activity and reducing potential.

Correlation between antioxidant activity and polyphenolic compounds: A positive, significant and linear correlation was found between total antioxidant activity and

polyphenolic contents (TPC & TFC) of various extracts. Correlation coefficient (R^2) values of different extracts showed a very close correlation between antioxidant activities and polyphenolic contents (TPC and TFC content). Positive and linear correlation (R^2, ranges from 0.982-0.998) was found in *A. paniculata* in the present experiment (Fig. 3). In the present work, we found a strong correlation between antioxidant activity and total phenolic contents (TPC & TFC). High correlation coefficient ($R^2 \geq 0.946$) values showed close correlation between them. Correlation coefficient (R^2) between antioxidant activity and polyphenolic contents (TPC & TFC) of aqueous and methanolic extracts of Chinese medicinal plant (Cai et al., 2004) and Jordanian plant species (Tawaha et al., 2007) are well reported.

Figure 2: Correlation between antioxidant and reducing potential in (A) Methanolic, (B) Ethanolic and (C) Aqueous extract of *A.paniculata* plants

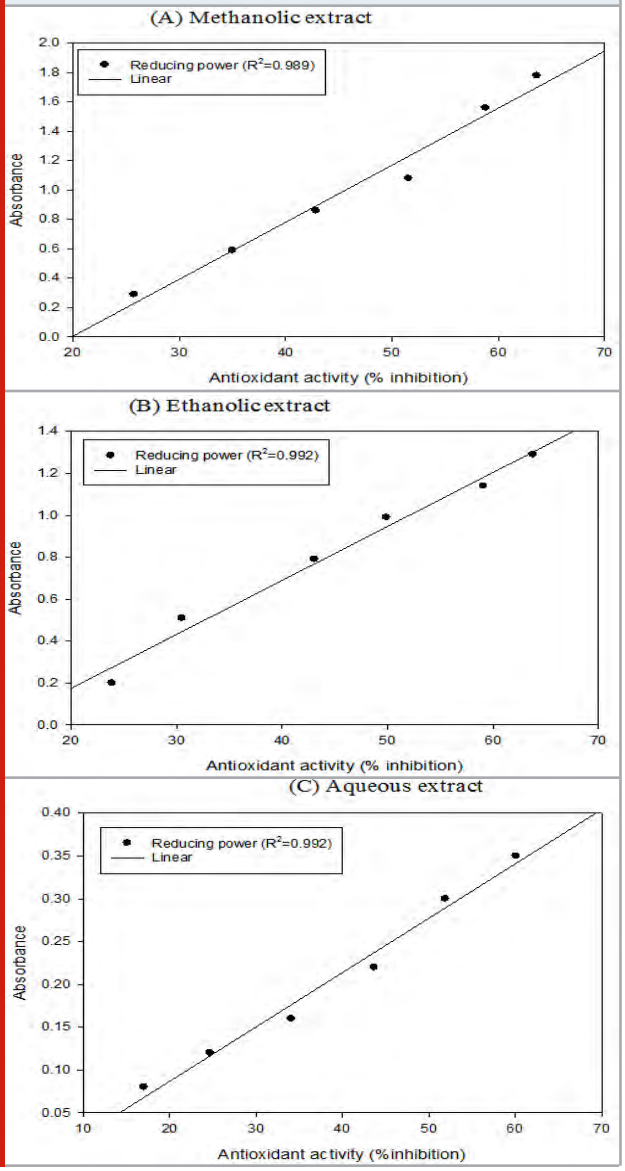
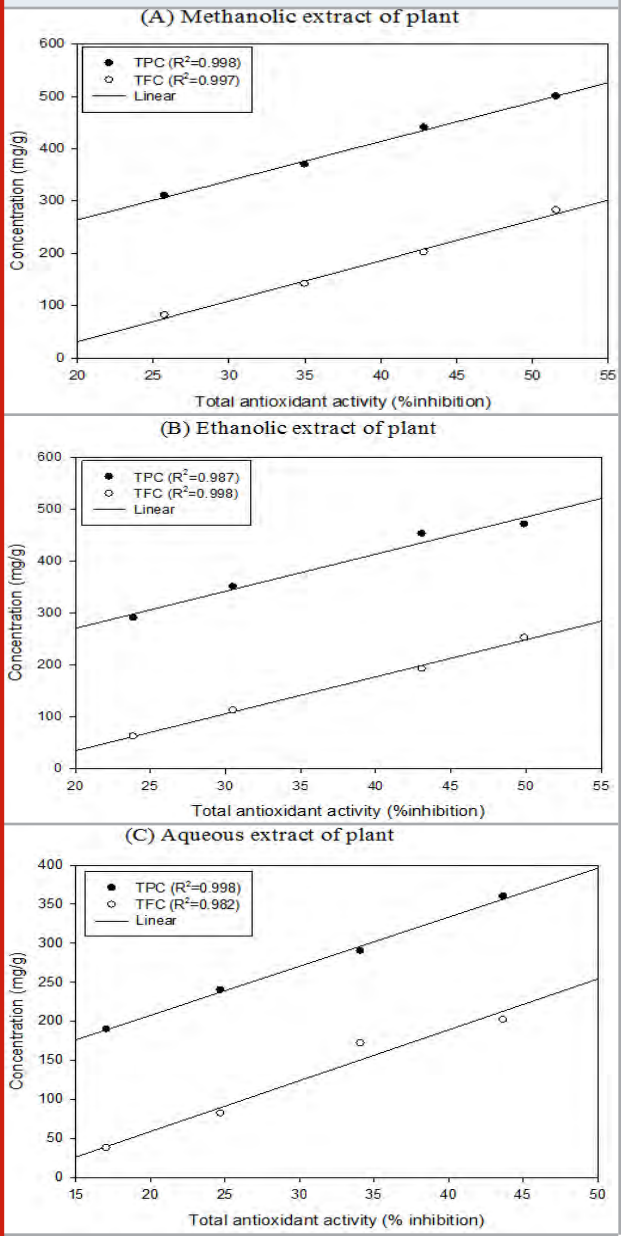


Figure 3: Correlation between antioxidant activity and polyphenols (TPC and TFC) (A) Methanolic (B) Ethanolic (C) Aqueous extract of *A. paniculata*



Phenolic compounds play an important role as antioxidants and a good correlation exists between the concentration of plant phenolics and the total antioxidant capacity (Sinha and Raghuwanshi, 2016a). The phytochemicals present in the plant and food products are generally nontoxic and contain many medicinal properties. Generally, antioxidants and polyphenolic compounds are mutually related with each other for their activities. *A. paniculata* is a good source of phytochemicals like phenolics, flavonoids, antioxidants, alkaloids and tannins etc. These phytochemicals play an important role in promoting pharmaceutical drug preparation and are used for curing various health ailments (Usman and Osuji, 2007).

CONCLUSION

Our study reports that the whole plant extract of *A. paniculata* plant is a rich source of natural antioxidants. The antioxidant property, reducing potential and polyphenolic components like total phenols and flavonoids varied significantly in the different extraction solvent. The organic solvent i.e. methanol and ethanol gave better results than aqueous one. Thus, bioactive compounds present in the extract of this plant may develop into antioxidant agents in the form of plant based drugs that may have applications in human health.

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Conflict of Interests: The authors declare that they have no competing interests.

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Fish Parasites as Biological Indicators: A Systematic Review

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ABSTRACT

The quality of the aquatic ecosystem has been severely degraded by anthropogenic activities over the last decade. The industrial wastes, effluents from paper mills, thermal effluents and run-off from agricultural lands act synergistically with the global climatic changes and alterations in seasonal cycles to affect the physico-chemical parameters of the water bodies and the life they sustain. Out of various impacts of the health of aquatic ecosystem on the environment, one tangible effect is definitely on the fishing industry. This emphasizes the importance of the study of bioindicators to estimate the extent of contamination of water bodies. Recently, various studies have highlighted the prospects of fish parasites as ecological indicators. Most of these studies include digenean trematodes, cestodes, nematodes and acanthocephalans parasitizing the fish gut. Ciliates and other protozoan parasites have also been studied, though not extensively. Endoparasites are more common in these studies because ectoparasites have been found to be more sensitive to changes in water quality parameters restricting their availability from polluted water bodies. Many endoparasites, especially the acanthocephalans, can acquire and store heavy metals in their bodies at concentration higher than those in host tissues. The parasites have been found to differ in their bioaccumulation and bioconcentration abilities and thus their bioindicator properties. They have also been found to act as indicators for eutrophication, acidification, hydrocarbons and other organic pollutants. As in every study in its nascent stage this too has certain limitations. Some of these include the complexities of the host-parasite relationships in a polluted environment, the taxonomic inter-relationships between them and the complicated physiology of metabolism of pollutants. The current review goes through the research studies undertaken in this field and tries to explore the potentialities of fish parasites to be used as bioindicators, analyse the constraints therein and highlight the thrust areas for future studies.

KEY WORDS: AQUATIC ECOSYSTEM, BIOINDICATOR, FISH PARASITE, POLLUTION.

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INTRODUCTION

The concept of parasite has changed in the recent years. Marcogliese (2005) rightly pointed out that parasites shouldn't just be treated as organisms that need to be eliminated. They are important and integral part of the ecosystem conveying information not only on environmental stress, trophic structure and function but also on biodiversity (Marcogliese, 2004). However, there

is a dearth of knowledge about the important roles of parasites in ecosystems (Marcogliese, 2004). Reportedly, the parasites serve as useful indicators of persistent ecological interactions since their life cycles clearly depict seasonal patterns and reflect the history of co-evolution between host-parasite relationships (George-Nascimento, 1987).

Parasites are useful indicators of ecosystem stability (Marcogliese and Cone, 1997). As Steedman (1994) had remarked that ecosystem health cannot be measured directly, study of the parasites and host-parasite system would serve a more important and effective way of monitoring ecosystem health. Fish parasites form an important part of the aquatic biodiversity and thereby show close co-relations with the changes of both the external and internal environment of the hosts (Palm, 2011). The changes invoked in the hosts may cause changes in the parasites both directly and/or indirectly (Palm, 2011). Pollution and stress have resulted in reduction of species richness of parasites (Marcogliese, 2004).

Some recent investigations (Yen Le et al., 2014; Bayoumy et al., 2015; Biswas and Pramanik, 2016; Gilbert and Avenant-Oldewage, 2017; Leite et al., 2017; Sures et al., 2017; Ashmawy et al., 2018; Naggar et al., 2018; Al-Hasawi, 2019; Mehana et al., 2020) point towards the manifold importance of the study of fish parasites as biological indicators. Indirectly they serve as parameters to assess the extent of damage caused by human activities as a result of overexploitation of the marine and/or freshwater bodies used as "dumping grounds" which combined together with the effects of climate change on the aquatic ecosystems sends a warning signal (Palm, 2011).

The relationship between the parasitic species and its environment is reportedly affected by almost 30 biotic and abiotic factors (Williams and Jones, 1994). This exemplifies the use of parasites in these studies. Lafferty (1997) described eight parameters to assess the environmental health based on parasite systems. This included heavy metals, acidification, sewage-sludge, industrial effluents, crude oil, pulp-mill effluent, thermal effluent, eutrophication and unspecified human disturbance. They studied mainly the Digenea, Acanthocephala, Cestoda, Monogenea, Nematoda and Ciliophora. Lafferty (1997) had mentioned that eutrophication caused an increase in parasitism whereas heavy metals and "unspecified human disturbance" caused a reduction in parasitism.

Attrill and Power (2002) reported the complexities of the combined effects of climate change and temperature on the ecology of host-parasite system. Marcogliese (2001) highlighted the consequences of climate change on the biotic and abiotic aspects of the aquatic ecosystem. Landsberg et al. (1998) aimed at developing a cost-effective index for monitoring and assessment of estuarine health depending on the fish-parasite ecology in combination with the changes caused by pollution. Studies have shown that marine ecosystems are much more stable as compared with that of fresh water ecosystems. As a result, the fish parasite diversity per host is higher in marine ecosystem (Palm, 2011). Marine parasites are thus equally important and have fewer drawbacks than the free-living animals as pollution-indicators (Marcogliese and Cone, 1997).

The current paper aims at the review of the recent trends in development of the use of this recent interdisciplinary approach involving the field parasitologists and ecotoxicologists as an important tool for monitoring and assessment of the health of aquatic ecosystem, its importance and prospects. In developing countries where the fishery industry is an important commercial aspect with millions being dependent on it, such studies are very important. Apart from this, the field also provides opportunities galore to devise cost-effective ways of monitoring the aquatic ecosystem.

Foundations of the use of fish parasites for monitoring and assessment of the aquatic environment: Pollutants and other environment manipulating factors may affect parasitism environment in both ways. Parasitism may be on the rise if the impact serves to reduce the host resistance or causes an increase in the density of the intermediate and/or definitive hosts (Lafferty, 1997). Other way round, parasitism may decrease if the impact results in the reduction of density of the intermediate and/or definitive hosts or it directly causes high mortality rates in the parasites because of the toxic effects or by indirectly causing the sufferance of the infected hosts and thereby the parasites (Lafferty, 1997).

The use of fish parasites as indicators is based on the fact that they are known to have life cycles that vary greatly in different species with one or more developmental stages infecting animals commonly used as markers or associated with the sea-sediments (especially the eggs and /or larvae) (Williams and MacKenzie, 2003). In case the free living stage(s) of the parasites are closely related to the sea bottom or sea sediments they alone can serve as indicators (White, 1984). These free-living infective stages of various parasites are delicate and highly sensitive to the surrounding environmental conditions enabling them to act as good indicators of pollution (Overstreet, 1993; MacKenzie, 1999). Free-living stages of parasites are also used in standard toxicity tests (Morley et al., 2003; Pietrock and Marcogliese, 2003).

MacKenzie et al. (1995) and MacKenzie (1999) suggested that the transmission stages of parasites, specially the

helminths could serve as early warning sign of marine pollution and was supported by the results of works carried out by Cross et al. (2001). Thus, sufficient knowledge on the biology of the parasites and their host as well as the host-parasite relationship is an important tool to detect environmental changes.

In case of heteroxenous parasites, the environmental conditions must be favourable at all stages of the intermediate hosts and the free living stage(s) of the parasite (if present) (Sures, 2008). However, in case of the monoxenous parasites only the environmental conditions of the host and the parasite should be taken into account (Sures, 2008). The density of these parasites in their respective host(s) may change if their life cycle is affected due to non-availability or poor availability of the intermediate hosts as a result of pollution (Palm, 2011). Thus, a study of the intermediate hosts and their availability at certain sites serve as important indicators for the occurrence of heteroxenous parasites in a polluted area (Overstreet et al., 1996).

The intermediate host(s) exposed to the environment directly may be more sensitive to changes while the endoparasites living within the hosts are prevented from such drastic changes and have a more or less stable existence (Paperna and Overstreet, 1981). Parasite species that are short lived and have direct life cycles like those of the ectoparasite protozoans or monogenean trematodes react to the direct environment of the hosts (Lester, 1990). Sures (2008) had opined that since the ectoparasites are in continuous contact with the water they may develop resistance to changes in water quality by alterations in their immune system (MacKenzie et al., 1995; MacKenzie, 1999). The outcome of such interactions is that the changing environmental conditions have less effect on monoxenous parasites as compared to that of heteroxenous parasites (MacKenzie, 1999).

Reports show that there is a trend in the increase of ectoparasitic infections and a decrease in endoparasitic infections with rising levels of pollution (Sures, 2005; MacKenzie, 1999). Reports of Khan and Kiceniuk (1983) and Khan (1987) show that decrease in the endoparasite infections with rising levels of pollution may be attributed to its negative effects on the intermediate hosts which in turn reduces infection in the definitive hosts. They had substantiated their reports using *Steringophorus furciger* (Trematoda) and *Echinorhynchus gadi* (Acanthocephala) as examples.

The parasite content of the fish may also serve as an important parameter for studying their feeding ecology and behavioural aspects (Klimpel et al., 2010; Palm, 2011). Parasites often represent an integration of several food chains, thereby forming an important part of the food web and many of them are in fact transmitted form one trophic level to another via intermediate host(s) (Marcogliese, 2004). Taking cue from this, one can successfully use the long lived life-cycle stages of the parasites and integrate the information with the detailed analysis of the stomach content of the fishes to derive information regarding the prey organisms which in turn helps in better understanding of the food web and other ecological aspects of the fish which would otherwise be difficult to study in some cases because they live under extreme conditions and are not easily accessible (Palm, 2011). Many workers have used the information to study the life cycles of many parasites like *Contracaecum* sp. and *Pseudoterranova* sp. (Klöser et al., 1992; Palm, 1999).

The impact of anthropogenic activity can also be deciphered form the biodiversity study of aquatic environments. For example, overfishing may cause a marked reduction (or even depletion under extreme conditions) of one or more species thereby reducing the biodiversity. This affects the parasite population adversely by affecting the availability of the intermediate host(s). Thus these studies are very important tools for assessment of environmental stress and the study of food web structures and biodiversity as shown by Marcogliese (2005).

The parasite populations may also serve as good indicators of climatic change since it is co-related with changes in ocean temperatures, salinity and circulation (Palm, 2011). Harvell et al. (2002) had demonstrated that climate change may affect the host-parasite interaction by augmenting the pathogen development rates, transmission or generation times annually or increasing the overwinter survival rate of the parasite or by raising the host susceptibility to thermal stress factors. This view has been supported by many time-based studies carried out by several workers like those of MacKenzie (1987) on the cestodes, *Grillotia angeli* (in mackerel) and *Lacistorhynchus* sp. (in herring).

Parasite species of certain digenean trematodes, cestodes or nematodes or any of their life cycle stage(s) that are long lived help in predicting the seasonal migration of the host species as well as the migratory behaviour of different life-history stages of the host (Williams et al.,1992; Palm, 2011). The original locality of the infection may be determined if there is sufficient knowledge regarding their abundance in different regions and the stages of hosts attacked by the parasites. Studies of Lühe (1910), Shulman (1957), Palm (2004), Klimpel et al. (2010) and Buchmann (2019) show the transfer of parasites or their dispersal by the migration of fishes from one region/part to another.

These reports also show that the parasites originating in the sea waters/ oceans may be transferred to the fresh water by extensive fish migrations.

Gibson (1972) could separate flounders (*Platichtys flesus*) form two estuaries and the sea based on dissimilar parasite faunas harboured by them. Thereby, the foreign flounder population migrating into these water bodies could also be identified by their set of parasite fauna. In another work, Margolis (1982) differentiated between three different spawning stocks of salmon, *Onorhynchus nerka* using the presence/absence of two species of myxozoans, *Myxobolus articus* in their brain and *Henneguya salmonicola* in the musculature. Such stock differentiations were helpful for in-season management of fishery (Moser, 1991). Recently this knowledge has been used by Lester et al. (2001) and Charters et al. (2010) to differentiate between the Spanish mackerel from Australia and Indonesia based on differences in the metazoan parasite content of these fishes. This is valuable information for the fishery industry for better utilization and sustainable harvest of the commercially important fishes (Palm, 2011; Buchmann, 2019). The details of these studies may be successfully applied to the native fish species of India or in any other country where the fishery industry contributes to national income.

Information regarding the parasite content of the fish species is equally important for identification of the parasites that may be transferred to the human beings via the fish musculature and may become a serious health issue (Palm and Overstreet, 2000). Study of such parasites of public health importance is another important aspect of the fisheries industry that should be taken care of. The reason for the abundance of such parasites at particular locations or ecological conditions (if observed) should be studied in details to develop control measures for them by simple manipulation of the environment of the fishes. Studies have demonstrated the prevalence and abundance of zoonotic parasites among different fish stocks from different geographical regions of the world (Palm, 2011). This forms the base for public health issue as well as for the thriving of tourism industry in a region (Palm et al., 2008; Klimpel and Palm, 2011).

The interplay between the effect of the pollutants on the immune system of the host and its reduced resistance to the parasites forms another important aspect of these investigations. Studies have demonstrated that exposure to immunosuppressive chemicals like heavy metals and PCB has negative effect on the immune system of the animals (Arkoosh et al., 1998; Bols et al., 2001). Immunosuppression caused by environmental pollutants might be one of the key-players in the successful establishment of the parasites (Sures, 2008). Some pollutants, mainly PAH, with immune-suppressive activities in the fish host affect the phagocytic activity either positively or negatively thereby affecting the intensity of gill-parasite infections (Williams and MacKenzie, 2003).

Such immune system based biomarkers may find important applications in assessment of water quality (Williams and MacKenzie, 2003). Hoole (1997) reviewed the effect of pollutants on the immune system of the host related to innate immunity, levels of antibody and leucocyte activity. He concluded that some of these factors of immune system may be augmented by certain pollutants which may in turn increase host-resistance to parasites. The opposite may also happen (Hoole, 1997). Studies should focus on immune evasion by parasites, evasion/tolerance of reactive oxygen intermediates, evasion of antibodies, proteinase inhibitor production and masking host proteins (Williams and MacKenzie, 2003).

The parasites not only affect the accumulation of the pollutants in the host but also their effects (Sures, 2008). Studies conducted by Baudrimont and De Montaudouin (2007) serve an important example. Their study demonstrated that the presence of digenean parasites in cockles exposed to cadmium (Cd) causes a decrease in the metallothionein concentration compared to uninfected individuals. There is a dearth of studies on the combined effects of the parasites and pollution with reference to the endocrine system of the hosts (Sures et al., 2006). Their analyses demonstrated an antagonistic relationship between the parasite and pollution. Eels infected with the swim bladder nematode, *Anguilicola crassus* exhibited increased concentrations of cortisol while the simultaneous presence of metal or PCB pollution or both caused a reduction in the levels of plasma cortisol. Same studies based on mammals however gave different and contradictory results. Generalised predictions on the effect of pollution and parasites on hormonal homeostasis of the host were found to be difficult and erroneous in some cases (Sures, 2008). This field requires a more exhaustive study before arriving at definite conclusions.

Another interesting aspect of the study of the parasites as biological indicators is its use in the identification of hosts, phylogenetic studies and also to decipher the systematic position (Rokici, 1983). Palm (2007) and Palm et al. (2009) devised a model system to study the ecology and co-evolution of the life-cycle of parasities in oceans using trypanorhynch cestodes. These works helped in the better understanding of the host switching of parasites (Palm et al., 2009) and the relaxed host specificity within certain groups of parasites (Palm and Caira, 2008). Analyses on the phylogeny of the deep-sea trematodes in fish by Bray et al. (1999) and nematode, *Anisakis simplex* in the whale hosts by Klimpel and Palm (2011) provide good examples of such studies.

Fish parasites as aquatic ecosystem indicators: The presence of certain parasites have been found to be closely co-related with some pollutants present in the water body. (i) Metals: Digeneans and acanthocephalans serve as good indicators of heavy metals and human disturbance (Lafferty, 1997). Experiments on acanthocephalans as accumulation indicators (Palm, 2011) include those on *Pomphorhynchus laevis* and *Paratenuisentis ambiguous* which demonstrated higher concentrations of cadmium (Cd) and lead (Pb) than their corresponding hosts (Sures, 2003; Sures and Siddall, 2003); *Aspersentis megarhynchus* with a higher heavy metal concentration (Ag, Co and Ni) than its host (Sures and Reimann, 2003) and many others (Mehana et al., 2020).

Cestodes have also been found to act as good indicators for heavy metals. Sures et al. (1997) reported a higher concentration of lead (Pb) and cadmium (Cd) from the tissue (posterior part of the proglottids) of the marine cestodes, *Bothriocephalus scorpii* than the tissue of the host, *Scophthalmus maximus*. Higher amounts of cadmium (Cd), lead (Pb) and zinc (Zn) was recovered from the tissue of *Carryophyllaeus laticeps* as compared to their cyprinid host, *Chondrostoma nasus* by Jirsa et al. (2008).

Barus et al. (2007) reported some nematodes like *Anguillicola crassus* and *Philometra ovata* also demonstrate higher heavy metal accumulations as compared to their fish hosts. Leite et al. (2017) studied the concentrations of thirteen different elements like lead (Pb), barium (Ba), aluminium (Al), magnesium (Mg), iron (Fe), copper (Cu), titanium (Ti), manganese (Mn), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd) and nickel (Ni) from the larval tissue of the nematode parasite, *Contracaecum* sp. and from the tissue of the fish host, *Acestrorhynchus lacustris*. The result showed that all of them, except one, had higher concentrations in the parasite larvae than in the host tissues (Leite et al., 2017). Eventually, it was concluded that the larvae of *Contracaecum* sp. could be successfully used for biomonitoring metal pollution because they could accumulate various essential, non-essential and toxic elements even during their larval stages (Leite et al., 2017).

Some intestinal parasites like the acanthocephalans have remarkable properties to bioaccumulate heavy metals in their tissues at concentrations as high as thousand times more than those of the host tissues (Mehana et al., 2020). However, these endoparasites vary in their abilities to bioaccumulate. Experiments performed with three groups of parasites, namely, trematoda (*Gyliauchen volubilis*), nematoda (*Procamalanus elatensis*) and acanthocephala (*Sclerocollum rubrimaris*) collected from the fish, *Siganus rivulatus* in the Red sea showed *S. rubrimaris* was a better bioindicator than the other two (Al-Hasawi, 2019). This was because *S. rubrimaris* had greater ability to bioaccumulate trace metals like cadmium (Cd) and lead (Pb) at concentrations higher than those of the host tissues (Al-Hasawi, 2019). Therefore, the bioconcentration factors of the parasite species determine their ability to act as good bioindicators of metal pollution (Al-Hasawi, 2019).

(ii) Hydrocarbons: MacKenzie et al. (1995) carried out extensive studies on the effects of marine pollution on two monogeneans *Diclidophora merlangi* (parasite on *Merlangus merlangus*) and *Dictyocotyle coeliaca* (parasite in *Raja radiata* and *Raja naevus*). Williams and MacKenzie (2003) highlighted their use as potential biomonitors of hydrocarbon pollution. Marcogliese et al. (1998) reported an increase in *Gyrodactylus* sp. infections in fishes as a result of increasing concentrations of PAHs (polycyclic aromatic hydrocarbons) and PCBs (polychlorinated biphenyls) in the aquatic environment. Study of the ratio of ciliates to digeneans in fish hosts serves an important index of the effect of crude oil in aquatic ecosystems (Lafferty, 1997).

Reports of Ruus et al. (2001) show that metacercariae of the trematode, *Bucephaloides gracilescens* from their host *Myoxocephalus scorpius* serves as an indicator for accumulation of lindane, a prominent environmental contaminant. The protozoan ciliophore, *Trichodina cottidarum* and *T. saintjohnsi* infecting *Myxocephalus octodecempinosus* have been reported to serve as important bioindicators for petroleum hydrocarbons present in the water (Khan and Thulin, 1991). Increased prevalence of the myxosporidia, *Ceratomyxa acadiensis* indicates rise in the levels of polycyclic aromatic hydrocarbons (PAHs) in the surrounding water (Khan, 1986). Increased PAH also results in the increased prevalence of monogenean trematodes (Khan and Kiceniuk, 1988).

Brázová et al. (2012) suggested that the acanthocephalan parasite, *Acanthocepahlis lucii* from perch intestines could acquire and accumulate PCBs (polychlorinated biphenyls) at concentrations higher than those of host tissues. The finding proposes the use of *A. lucii* as bioindicators of hydrocarbon pollution (Brázová et al. (2012). From studies conducted on larval stages of the parasitic cestodes, *Oncomegas wageneri* collected from the intestine of their fish hosts, *Cyclopsetta chittendeni* it was concluded that the concentrations of PAHm (polycyclic aromatic hydrocarbon metabolites) was higher in the larval cestodes than in the host bile (Soler-Jiménez et al., 2020). Therefore, they can be used to biomonitor PAH levels in the surrounding water (Soler-Jiménez et al., 2020).

Investigations on concentrations of PAHs like chrysene, dibenzo(a,l)pyrene, dibenzo(a,i)pyrene, benzo(g,h,i)

perylene and indo(1.2,3-cd)pyrene among others from the parasitic trematode, *Aspidogastrea africanus* and their fish hosts, *Chrysichthys nigrodigitatus* showed the parasites absorb considerable amount of PAHs lowering their concentrations in their host (Akinsanya et al., 2020). Thus, they may be used as bioindicators to monitor PAHs in the water body. Additionally, it was also reported that the parasites preferred to infect female hosts and caused oxidative stress in their intestines (Akinsanya et al., 2020).

(iii) Eutrophication/Paper mill effluents/Thermal effluents: Ciliates and nematodes serve as sensitive indicators of eutrophication and thermal effluents(Lafferty, 1997). Barker et al. (1994) reported increased levels of the digenean parasite *Cryptocotyle lingua* in sites contaminated by pulp and paper mill effluents. Density of *Diplostomum* sp. has been reported to be an indicator of increasing eutrophication (Hartmann and Nümann, 1977). Other reported parasite bioindicators for eutrophication includes the acanthocephalan, *Echinorhynchus* sp.; the nematode, *Ascarophis nototheniae*; the trematode, *Dinosoma* sp. and the cestode, *Phyllobothrium* sp. (Moser and Cowen, 1991). Though there is a dearth of information regarding suitable fish parasite indicators for eutrophication and other effluents, studies by Johnson and Chase (2004) and Johnson et al. (2007) show that higher levels of eutrophication promotes higher prevalence of trematode infections in frogs because of the increased abundance of the intermediate snail hosts. Thus, this field has scope for further studies.

(iv) Acidification: Acidification has the most notable effects on population of digeneans (Marcogliese, 2005). Presence or absence of digeneans in fishes serve as indicators for assessment of the impact of acidification in the water sheds because acidification causes a reduction in the population of the intermediate host snails (Kurris and Lafferty, 1994). MacLeod and Poulin (2012) concluded from their experiments that parasites could be used as bioindicators of ocean acidification (OA) disturbance. In their further studies they had expressed their anxiety over the rising ocean acidification and its effect on productivity, biodiversity and ecosystem health (MacLeod and Poulin, 2016).

Their investigations showed that the presence of parasites affects the sensitivity of their hosts to ocean acidification. The death of the infected gastropods was significantly lowered under laboratory conditions imitating the circumstances of OA (MacLeod and Poulin, 2016). They had recommended further research on the area. Harland (2015) had mentioned the use of intertidal parasites as good bioindicators for assessing the impacts of ocean acidification. Harland (2015) had also suggested that ocean acidification may affect the transmission of parasites.

(v) Organic pollution/Bacterial biomass: According to the reports of Ogut and Palm (2005) *Trichodina* spp. act as indicators of organic pollution depicting the concentrations of nitrate, nitrite and phosphate in aquatic environment. Chubb (1997), Yeomans et al. (1997) and Yoemans et al. (1999) carried out investigations and discovered a link between the bacterial load of water bodies and increased trichodinid infestations in fishes. Palm and Dobberstein (1999) reported that *Trichodina* spp. also acts as an important bioindicator of bacterial biomass in the environment as they are known to be filter feeders with bacteria and algae forming important parts of their diet. Investigations by Yen Le et al. (2014) show that the parasites can not only bioaccumulate metals and heavy metals but also POPs (persistent organic pollutants) by virtue of the lipophilic nature of these molecules. The accumulation factor of these organic compounds is affected by certain biotic and abiotic factors as well (Yen Le et al., 2014).

Limitations of the use of fish parasites as indicators: The studies based on host-parasite system are not free from restrictions. Some of these include:
(i) Host-parasite relationships in a polluted environment: The host-parasite relationships in a polluted environment is highly complicated. For example, from the studies on roles of parasite in accumulating heavy metal(s)/other pollutant(s) it may appear that the reduced concentration of the pollutants in the host may be beneficial for the infected animals. It may thus be predicted that an infected animal when exposed to environmental pollution, the presence of parasite may be advantageous for them as the pollutants tend to accumulate more in the parasites harboured by them rather than the host themselves (Sures, 2008). This may seem to have a shielding effect from the toxic reactions of the pollutants when compared with the uninfected animals. However, this is not such a simple process and before such assumptions can be made, this field needs more studies to check whether the deleterious effects of the parasite on the host can be overlooked by the advantage it enjoys from the shielding effect (Sures, 2008).

(ii) Relationship between environmental factors and parasites: Lafferty (1997) had suggested that there was a weak association between parasites and environmental degradation because environmental factors had different effects and parasites responded differently complicating the whole scenario. Kennedy (1997) opined that fish parasites could be used as bioindicators only after the relationship between pollution and fish parasite have been studied in detail otherwise it would not be advantageous to use them over free living organisms. Marcogliese (2005) suggested that parasite communities do not serve as good pollution indicators in case of moderate levels of contaminants.

(iii) Lack of knowledge on ideal conditions for a fish parasite to qualify as a bioindicator: Vidal-Martínez et al. (2010) opined that the definite conditions under which a parasite may be considered to act as indicators of environmental change or impact becomes very difficult in large scale considerations because too many factors are involved and the study of such factors individually offers various complications. Catttadori et al. (2005) suggested that climate change events may lead to outbreak of parasitic infections which may cause dramatic, synchronized abundance of their hosts citing the nematode parasite, *Trichostrongylus tenuis* affecting red grouse as example. Studies of Kennedy et al. (2001) based on the larval cestode, *Ligula intestinalis* and roach hosts (*Rutilus rutilus*) suggested that parasite-host systems can also regulate the population structure of the ecosystem rather than climate change alone. Whatever may be, this field is rather naïve and needs more elaborate studies before making generalised comments.

(iv) Variability in response of the parasites to external changes: Parasites respond to environmental changes in various ways making generalized predictions extremely difficult. For example, Gheorghiu et al. (2006) reported an increase in the parasite population of *Gyrodactylus turnbulli* with the increase in concentration of zinc upto 120g/l in the surrounding water while an increase of zinc concentration beyond this may cause a reduction in the population of *Gyrodactylus turnbulli* (Gheorghiu et al., 2007) as it adversely affects the growth and reproduction of the parasites. A study by Gilbert and Avenant-Oldewage (2017) showed the sensitivity of oncomiracidia of *Paradiplozoon ichthyoxanthon* to dissolved aluminium (Al) under laboratory conditions. They had suggested that the parasites from both the marine and freshwater ecosystems in Africa could serve as bioindicators but the effects of the pollutants on those organisms needed to be studied in details. Such reports make it very clear that simple prediction between prevalence of parasite population and increase in concentration of a particular pollutant may be erroneous and needs an elaborate study.

(v) Difficulties in community-based monitoring of parasites: Studies based on single measures of parasite communities, like species richness, as indicators of specific environmental changes are not scientific and often erroneous because different taxonomic groups tend to respond in opposite directions (Lafferty, 1997). Community-level monitoring serves a more important tool for exhaustive assessment of environmental change and requires deeper insights in the association between the wide range of parasites and impacts (Lafferty, 1997). This requires both laboratory-based studies and field-based studies. There is however, a dearth of studies that combine field and laboratory evidences together (Lafferty, 1997). The effect of pollutants on the developmental stages of the parasites like the cercariae and miracidia of digeneans may also affect the parasitic communities and needs to be further investigated as suggested by Lafferty (1997). Most parasites are species specific being restricted to certain host species but there are some which infect a wide range of hosts. This sharing of parasites by the hosts affect the structure of parasitic communities in aquatic bodies (Buchmann, 2019) and any factor (including a pollutant) affecting this behaviour may interfere with parasitic communities. Such altered interactions may also affect the metabolism pathways of various pollutants as well as their toxic effects. This calls for further research in this field.

DISCUSSION

Sures (2008) had rightly remarked that the combined effects of the parasites and pollution on the host is very complicated and may be additive, synergistic or antagonistic making predictions extremely difficult. The gravity of this line lies in the fact that a parasite alone cannot be simply used as a bioindicator until and unless it is clear which stage of the parasite qualifies for the purpose and the myriad of factors affecting it. This is because there may be one to several free-living stages of a fish parasite that are exposed to the surrounding medium directly and the sensitivity of these stages (Poulin, 1992; Valtonen et al., 1997) to different concentrations of the pollutants need to be documented over a long period of time (Williams and Mackenzie, 2003) to understand how the dynamics of the parasitic population in the water bodies is affected by contamination. Since the behaviour of the parasite population varies with the pollutants, entire parasitic community in a water body may be affected.

This area has contradictory reports. While some studies claim that the population of the fish ectoparasites increases due to pollution (Khan and Thulin, 1991; Sures, 2005; Biswas and Pramanik, 2016) there are some studies which insist that the ectoparasite population decreases considerably because of their high sensitivity to the contaminating agents (Gilbert and Avenant-Oldewage, 2017). There maybe two explanations for this variation in results – first that the parasite species being considered in both the studies are different and the second one is probably the length of the free-living stages of the parasites in question. It cannot be negated that the duration for which the ectoparasites are exposed to the pollutants is sufficiently higher than those of the free-living stages of the endoparasites.

The shorter the length of the free-living stage the lesser is the effect of pollutants on it eventually resulting in increased parasitic burden of the final host. Another thing that needs consideration is the immunological reactions of both the ecto and endoparasites that allows

them to survive the harmful effects of the pollutants. Sures (2008), however, suggested that ectoparasites probably develop immunological resistance to fluctuating environment due to their long periods of exposure. Immunity studies becomes particularly important in endoparasites because their free-living stages should be having some special mechanisms to evade the responses to enable them to survive over the ectoparasites under similar circumstances. Studies should be encouraged in these fields to enable the workers to understand how the system works and rethink the prospects of parasites being used as bioindicators.

Another aspect that seems to be neglected is the study of the effect of pollutants on intermediate hosts (Khan and Kiceniuk, 1983; Khan, 1987) because the continuity of the life cycle of the parasites demands the availability of the intermediate hosts. The pollutants may therefore affect the parasitic communities of an aquatic ecosystem mainly by three ways – a) exerting their direct effects on the developmental stages of the parasite, b) exerting their effects on the intermediate hosts affecting their availability and c) affecting the susceptibility of the definitive hosts to be affected by the parasites. Then, there is a fourth way by which the pollutants may impact the sharing of parasites between the available definitive hosts in the water body. All of these need further examination before drawing conclusions. Studies like those of Johnson and Chase (2004) and Johnson et al. (2007) should be encouraged.

From all the available literature it seems that the endoparasitic helminths have emerged as the candidates of choice for biomonitoring aquatic bodies for metal pollution. The results with acanthocephalans have been found to be particularly promising. Various hypotheses have been proposed with respect to the presence of metals in higher concentration and detectible amounts in these helminths than the host tissues like intestine, muscles and liver (Leite et al., 2017). According to some studies the sites of location of the parasites within the hosts affects the concentration of metals (Moravec, 1998).

The preferred locations include the stomach or intestine before they move on to the muscles or other organs (Moravec, 1998). The bile acids have been proposed to react with the metal ions within the digestive tract of the fish hosts resulting in the production of organo-metallic complexes (Mehana et al., 2020). These organo-metallic complexes are lipophilic substances that can be easily taken up by the helminths resulting in their high concentrations within their body (Mehana et al., 2020). Another hypothesis states that the bioaccumulation properties of helminths depend upon their developmental stages.

Comparative studies on accumulation properties of metals using adults and larvae of *Anisakis simplex* showed that the larvae had higher accumulation rates of cadmium (Cd), zinc (Zn), lead (Pb) and copper (Cu) than those of the adults (Pascual and Abollo, 2003). As an explanation for this observation it had been stated that the less complex nature of the larval cuticle enabled the adsorption of metals across the body surface (Leite et al., 2017). Studies in these fields must be encouraged because there is a dearth of sufficient data to understand the bioindicator properties of the helminths. Moreover, information on effects of other pollutants like hydrocarbons, thermal effluents, effluents from paper mills, pesticides and sewage on ecto and endoparasites are insufficient and need to be worked out.

Despite the detrimental effects of PAHs (polycyclic aromatic hydrocarbons) and PCBs (Polychlorinated Biphenyls) on the aquatic ecosystem the area has rarely been studied resulting in scanty information over the subject. The far-reaching effects of hydrocarbon pollution include alterations in benthic communities to biodiversity erosion to even modified feeding activities of the aquatic organisms (Ellis et al., 2012). It has been found that marine fish species take up PAHs when exposed to oil spills (Soler-Jiménez et al., 2020). Earlier studies show that the fishes and other aquatic vertebrates after absorbing the aromatic hydrocarbons from their environment metabolize the PAHs by various pathways and give rise to PAHm (polycyclic aromatic hydrocarbon metabolites) which are excreted through bile (Soler-Jiménez et al., 2020). The bile of these animals can thus be analysed for the presence of PAHm and their concentrations can be compared with PAHm concentrations of the endoparasites harboured by them (Soler-Jiménez et al., 2020). It needs to be mentioned here that the parasites have their own pathways to metabolize PAH and produce PAHm (Soler-Jiménez et al., 2020).

The studies are indeed intriguing but the most interesting part is that parasites, especially intestinal endoparasites, have lipid concentrations lower than their corresponding hosts (Yen Le et al., 2014). Despite this, the lipophilic substances including PAHm have been found in higher concentrations in the parasites than their host (Soler-Jiménez et al., 2020). A few hypotheses have been proposed for such peculiar observation. One such hypothesis proposes that the parasites are not very efficient in eliminating the PAHm like their hosts resulting in these high molecular weight compounds being stored within the parasite body (Soler-Jiménez et al., 2020).

Another hypothesis suggests that the parasites probably produce higher amounts of PAHm compared to their hosts (Soler-Jiménez et al., 2020). A third hypothesis

suggests that population density of the parasites lodged within their host's intestine affect their uptake of lipophilic substances from the surroundings impacting the concentrations of PAHm in both the parasites and the host (Soler-Jiménez et al., 2020). Needless to mention that sufficient data to support any of these hypotheses are not yet available though there is a probability that all of these might be true. This area needs special attention.

CONCLUSION

This field is naïve and a revolutionary one that has broadened the scope of parasites beyond simply "disease causing organisms." The concept of parasites as "superorganisms" (Marcogliese, 2005) is evolving gradually. Studies of parasites from this aspect give many important insights regarding their ecological demands and/or behaviour. For example, recovery of parasite communities after decrease in chemical and nutrient loads in the aquatic ecosystems has been documented by Cone et al. (1993) and Valtonen et al. (2003). The choice of the parasite(s) and/or host-parasite system is crucial to these studies and the set of guidelines summarized by Williams et al. (1992) is indeed a landmark for workers in this field.

Literature from all aspects show that the maximum volume of studies in this field has been restricted to metal pollution. Despite the deleterious effects of other pollutants on the aquatic ecosystems and environmental health effective techniques to biomonitor these compounds using parasites have been poorly studied. A few recent studies, however, have exhibited encouraging results and needs further research. Considering the deplorable condition of the current ecosystem health this may go a long way in understanding the shocking and far-reaching impacts of anthropogenic activities.

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Identification and Bioinformatic Analysis of 16s rRNA Gene Sequences of Native *Bacillus* sp. isolated Strains from Saudi Arabia

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ABSTRACT

36 Bacillus native strains were isolated and identified using 16s gene sequencing analysis. Universal primer of 16s rRNA gene was used and PCR amplifications were done with all DNA isolation from all isolated strains. Mixture of non-identical 16S rRNA amplicons products produced and bioinformatics data analysis of all yielded variable consensus sequence were done using different software programs. The data analysis of sequences displayed some sequence variability occurs among the multiple 16S rRNA genes. Bioinformatical analysis revealed that the nucleotide sequences obtained from the isolates were different homologous to the known DNA sequence using NCBI Blast software. Results revealed that the obtained Bacillus isolated strains were belonging to17 different species. No noticeable base frequencies variation were observed in base frequencies for all the 39 isolates except B.simplex strains in in cytosine base. Results of phylogenic analysis revealed that all isolated strains are clustered analysis in 2 large clusters. The Polymorphism and Genetic Diversity among the isolates were also done using Clustalx and Maft build analysis, the monomorphic and polymorphic sits of all strains also analyzed.Further study for complete genome sequence analysis will be needed. The bioinformatics tools were succeeded in analyze all our native bacillus strains.

KEY WORDS: BACILLUS. DNA, 16S RRNA, GENES, BIOINFORMATICS ANALYSIS.

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INTRODUCTION

The microbes in the rhizosphere are a diverse mixture of microorganisms that can actively interact with the host plant in different ways. The studies on the microbial rhizosphere communities have shown the significant influence of plant species and cultivars in shaping microbial communities in the rhizosphere (Lei et al., 2018).Soil is an important environment and contain all number of additives such as mineral with organic

elements for plant growth, also contain huge number of microorganisms safe and non-safe for plant (Wołejko et al., 2020). The soil conditions are important for plant and microorganisms such as physical respectabilities and chemical constituents (Tajik et al., 2020). Many microorganisms isolated and identified from soil all over the world by scientist's, such as bacteria, algae, fungi, etc. all these microorganisms are benefits for plant production and, also fight plant pathogens. So, it plays a main role for biological control of plant pathogens. Significant characters from soil bacteria such as gibberellic acid (GA3), indole-3-acetic acid (IAA) which are phytohormones, nitrogen fixation, and nutrient solubilization show main factors in plants production (Tabatabaei,et al., 2016, Wołejko et al., 2020).

Bacillus strains have been extensively studied specially *Bacillus thuringiensis* because of its huge benefits and safe for human being. There are too many applications of these bacillus under study in biological control because it used for fight many types of insects (Sanahuja,et al., 2011). *B. amyloliquefaciens, B. subtilis, B. megaterium* and *B. thuringiensis* and other similar bacterial strains isolated from soil types when microbiome in soil studied by (Boottanun et al., 2017; Kui et al., 2019; Panneerselvam et al., 2019). Isolation of bacillus strains and species from soil is easy way, it isolated from various depth (Labo et al., 2018: Kui et al., 2019).

The 16S rRNA gene sequence is used for phylogenetic studies based on the produced sequences (Woese et al., 1977). Bacterial 16S rRNA genes are located within the rRNA operons, which also contain genes for 23S rRNA, 5S rRNA, tRNA, and associated intergenic spacer regions (Woese et al., 1990). Identification of bacterial strains to the genus and species level is preferred according to sequencing of rRNA genes because this gene contain highly conserved and hypervariable regions (Anda et al., 2015). 16S rRNA sequence analysis used for identification of bacterial isolates this method is reliable and fast than classical conventional methods. (Woo et al., 2008). Phenotypic characteristics analysis including Gram staining, cell morphology, and biochemical properties are still the first steps in species identification, and 16S rRNA sequence analysis may be performed only when the phenotypic results are not definitive (Clarridge. 2004).

In the genome of some single strains there are variation in 16S rRNA gene sequences such as the presence of single nucleotide polymorphisms (SNPs) or small insertions or deletions among the multiple 16S rRNA copies (Sacchi et al., 2002;Acinaset al., 2004). By universal 16S rRNA designed primers to target conserved sites and targeted the 16S rRNA gene in isolated DNA from any bacterial strain and sequencing of such amplified products, now useful method for bacterial identification (Petti,et al., 2015). In this study isolation and molecular identification of some bacillus strains from Saudi Arabia soil were done. Bioinformatics analysis and comparison of multiple 16S rRNA genes sequencing from isolated bacterial strains were done.

MATERIAL AND METHODS

Four different soil samples were collected from different regions of Jeddah City. Isolation of Bacillus strains were done. 0.5g of soil samples was suspended in 10 ml of sterile distilled water, soil samples was homogenized vigorously with vortex. After mixing 1 ml of the supernatant was stored at80oC for some minutes to kill most non-spore-forming bacteria.100 µL of suspension spread over nutrient agar plates. The apparent colonies were spread on the entire surface of nutrient agar m plates with triplicate. After incubation at 30oC for two days all colonies like bacillus colonies were chosen. The obtained single colony re-purified by re-streaking over Nutrient agar plates several times, stored in Nutrient agar slants at 4oC, and long term storage in Nutrient agar broth with 30% glycerol at -80oC (Masoud et al., 2015; Al-Yahyawy et al., 2019).

Extraction of DNA: Extraction of DNAs was carried out by I mL of each isolated strains was centrifuged to collect the pellet. 200µL of TES buffer (50mM EDTA, 100mM Tris pH8, and 10% SDS) and 20µL Lysozyme added to the bacterial pellet with gentle pipetting at 37oCfor 60 min., 20µL of proteinase K solution added to the mix incubated at 37oC for 60 min., the pellet suspension transferred to ice path for 5 min. then 250 µL of Sodium acetate 4 Madded. The mix was spin down for 5 min. at 10.000 rpm for 5 min. clear supernatant was transferred to a new tube and 250 µL of chloroform/isoamyle alcohol added to the supernatant in ice path for 5 min. centrifugation was done at 10.000 rpm for 5 min. the collected DNA pellet dried for 10 min in laminar flow cabinet. 50 µL of TE (10mM Tris pH8, 1mM EDTA) added to each tube. The DNA were detected by electrophoresis in 1.5% agarose gel (Sabir, et al., 2013; Al-Yahyawy,et al., 2019.).

16s rDNA isolation and analysis: PCR amplification and sequencing of 16S rDNA16S gene sequencing and analysiswere done. The amplified PCR products of the 16S rRNA gene bacterial gene fragments were detected by electrophoresis in 1.5% agarose gel. The amplified fragments were purified and sequenced at MACROGEN sequencing company, Seoul, Korea using the automated sequencer ABI 3100 (Applied Biosystems) with Big Dye Terminator Kit v. 3.1 (Applied Biosystems). Primers 518 F (5' C CA GCAG CC GC GG TAATACG - 3') and 800 R (5'- TA CC AG GG TA TCTA AT CC -3') were used for sequencing. The sequences obtained were edited with the software Vector NTI Suite 9, and compared with the NCBI database through BLAST searches. In this comparison, sequences of type strains most closely related to the sequences of the isolates were searched. For the definition of operational taxonomic units (OTUs), a similarity limit of 97% was adopted (Sabir, et al., 2013; Al-Yahyawy,et al., 2019).

Bioinformatics and data analysis: All the 39 sequences were subjected to ncbi BLAST search tool http://blast.ncbi.nlm.nih.gov to detect non-chance sequence similarity. BLAST search was restricted to 16S ribosomal RNA (Bacteria and Archaea), where models (XM/XP) as well

as unclultured/environmental samples were also filtered out, such that more reliable results would be attained. Each individual sequence was solely blastd, where blast hit with the lowest expect-value (which indicate number of non-chance alignments) was picked. In order to ensure that Blast out puts were governed by expected-value (aka e-value), Blast algorithm parameter was decreased such the expected threshold was set to more stringent value of 1e–6. Alignment of the 39 sequence was carried out using version 2 of Clustalx (Larkin et al., 2007).

Exploratory data and phylogenetic analyses were carried out under R Project for Statistical Computing (R Core Team, 2017). Where Exploratory data analysis was done using Seqinr (Charif&tLobry, 2007) R package. Phylogenetic analysis was carried out by ape package (Paradis, et al., 2004). Reconstruction of the phylogenetic tree was done using Neighbor joint method (Nei, 1987). DnaSP (LibradoandRozas, 2009) software was used to analyze the haplotype diversity (Hd), the average number of nucleotide differences, the average number of nucleotide differences (Tajima, 1983), the nucleotide diversity (π). The polymorphic site (S), the singleton variable sites (SP), and the parsimony informative sites (PIP) for each gene, and the average number of nucleotide substitutions per site between species (Dxy) (Lynch and Crease, 1990).

RESULTS AND DISCUSSION

Bacterial samples: Out of 36 Bacillus bacteria from soil samples near Jeddah were isolated on nutrient agar plates as pure culture in this study(Table 1) and fig. (1).The isolated strains were re-purified and streaked over the same plates (Fig. 1). 16s rDNA gene of the isolates were done as shown in fig (2). The16S rRNA were identified by 16S rRNA sequence analysis at Macrogen Company Korea, with Macrogen universal primer as identified in materials and methods. The results of 16 s gene isolation as shown pure 16S rRNA gene isolation in fig (2).

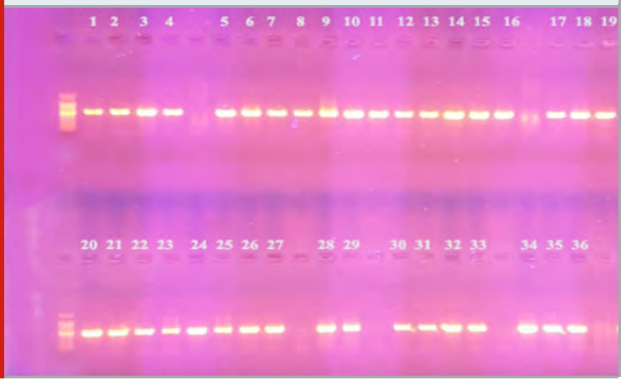
Figure 1: An example for purification of Bacillus isolates appeared over nutrient agar plates.

NCBI BLAST query: BLAST results are shown in Table (2) for all the 39 isolates. The criteria used for query sequence aimed to narrow down the search space (database), as the smaller the database the more likely to contain sequence of interest. For all the 39 queries zero E-values were attained indicated that all alignments were non-chance alignments. The query cover percentage ranged from 93 to 100 % with the only one exception for isolate 33. The vast majority of values of identical percent (% of how similar the query sequence is to the target sequence) ranged from 97% to 100% with only 2 isolates have identical percent of 80% and 82% table(2).

Table 1. Soil Samples Locations

Sample number	city	location
1	Tabuk	28.378089,36.565889
2	Baljurashi	19.865853,41.582135
3	Khobar	26.213500,50.188347
4	Jeddah	21.490086, 39.202683

Figure 2: 1 16S rRNA isolation on agarose gel electrophoresis

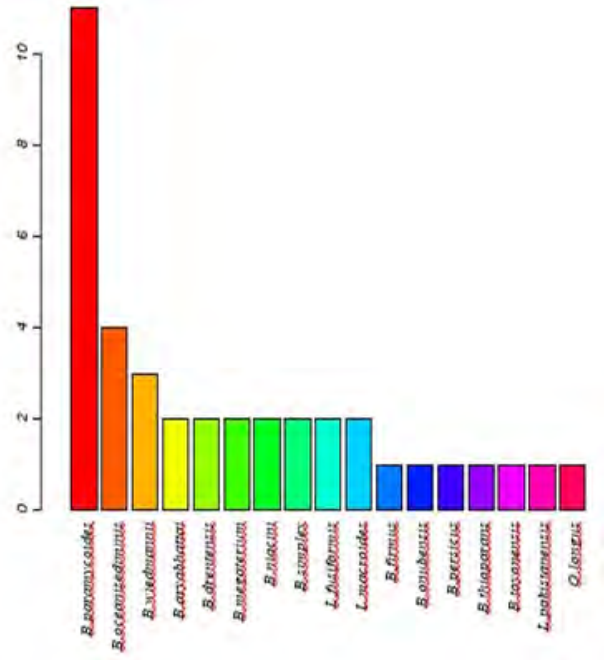


The distribution of isolates/species/distribution is presented graphically in Figure (3)the NCBI BLAST query resulted in 17 species, mostly belong to Bacillus sp,namely Species number 11 *B.paramycoides, 4 B.oceanisediminis,3 B.wiedmannii, 2 for each of B.aryabhattai, B.drentensis, B.megaterium, B.niacini, B.simplex, Lysinibacillusfusiformis* and *Lysinibacillusmacroides*, where only one isolate was found to be belong to each of *B.firmus, B.onubensis,B.persicus, B.thioparans, B.toyonensis, Lysinibacilluspakistanensis* and *Oceanobacilluslongus*.

Exploratory data analysis: Table (3) shows a description of the isolated species as well as their sequence length and the percentages of GC content of each isolate. Sequence length varied greatly among the 39 isolates ranged from 463 bp for *B. oceanisediminis* to 500 bp for *B. onubensis*. The percentage of GC content were found to be constant within species with no similar trend was observed for the Saudi isolates. Generally speaking, % GC ranged from 52 for B.megaterium to 56 for *B.persicus*. Base frequencies for all the 39 isolates is shown graphically in figure (4)No noticeable variation

were observed in base frequencies for all the 39 isolate exceptfor *B.simplex* where this species has a noticeable access in cytosine base.

Figure 3: The distribution of isolated bacterial strains at species level



Cluster & phylogenetic analysis: Cluster analysis and heatmap were carried out as pre-processing step to glean an insight into the data distribution. Results of cluster analysis are shown graphically in figure (5), where 2 large clusters are shown. The first cluster comprises merely 2 species *B.simples* and *Oceanobacilluslongus*, which indeicate that these two speices are distantly densely related to the rest of species. All other 37 species were clurested together in the second cluster occupying different clades.

Evolutionary relationships of taxa was inferred by reconstructing phylogenetic tree using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 1.2 is shown (figure 6). The results of phylogenetic analysis deeply sports the findings of cluster analysis. *B.simples* and *Oceanobacillus longus* were found to have the longest branches of the phylogenetic tree, which again indicate that these two species are distantly densely related to the rest of species. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method are in the units of the number of base substitutions per site. The analysis involved 39 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 437 positions in the final dataset.

Table 2. Blast results of isolated Bacillus strains

Isolate	BLAST Results			
	Species	Query cover%	E-value	% identical
11-27F	*B.paramycoides*	100	0.00	99
12-27F	*B.paramycoides*	100	0.00	100
13-27F	*B.paramycoides*	99	0.00	100
14-27F	*B.paramycoides*	100	0.00	99
15-27F	*B.paramycoides*	100	0.00	99
27-27F	*B.paramycoides*	99	0.00	100
32-27F	*B.paramycoides*	100	0.00	100
34-27F	*B.paramycoides*	99	0.00	100
36-27F	*B.paramycoides*	98	0.00	99
46-27F	*B.paramycoides*	99	0.00	100
6-27F	*B.paramycoides*	99	0.00	100
30-27F	*B.oceanisediminis*	95	0.00	99
37-27F	*B.oceanisediminis*	93	0.00	99
49-27F	*B.oceanisediminis*	95	0.00	99
29-27F	*B.oceanisediminis*	96	0.00	99
C2-27F	*B.wiedmannii*	99	0.00	100
18-27F	*B.wiedmannii*	99	0.00	100
10-27F	*B.wiedmannii*	100	0.00	99
19-27F	*B.aryabhattai*	99	0.00	100
20-27F	*B.aryabhattai*	99	0.00	100
2-27F	*B.megaterium*	99	0.00	100
8-27F	*B.megaterium*	99	0.00	100
22-27F	*B.simplex*	100	0.00	99
28-27F	*B.simplex*	97	<0.00	82
43-27F	*B.niacini*	99	0.00	99
5-27F	*B.niacini*	99	0.00	98
31-27F	*B.drentensis*	100	0.00	99
4-27F	*B.drentensis*	100	0.00	97
40-27F	*Lysinibacillus fusiformis*	100	0.00	98
41-27F	*Lysinibacillus fusiformis*	100	0.00	98
50-27F	*Lysinibacillus macroides*	99	0.00	99
52-27F	*Lysinibacillus macroides*	99	0.00	99
1-27F	*B.onubensis*	95	0.00	99
44-27F	*B.firmus*	100	0.00	97
45-27F	*B.thioparans*	99	0.00	98
48-27F	*Lysinibacillus pakistanensis*	99	0.00	96
9-27F	*B. persicus*	99	0.00	99
C1-27F	*B. toyonensis*	99	0.00	99
33-27F	*Oceanobacillus longus*	72	<0.00	80

Table 3. The sequence length and percentage of GC ratio of isolated *bacillus* strains

Species	sequence Length	GC%
12-*B. paramycoides*	474	0.53
15-*B. paramycoides*	471	0.53
32-*B. paramycoides*	476	0.53
46-*B. paramycoides*	476	0.53
27-*B. paramycoides*	467	0.54
13-*B. paramycoides*	477	0.53
36-*B. paramycoides*	476	0.53
6-*B. paramycoides*	476	0.53
11-*B. paramycoides*	479	0.53
14-*B. paramycoides*	478	0.53
34-*B. paramycoides*	475	0.53
29-*B. oceanisediminis*	463	0.55
30-*B. oceanisediminis*	472	0.55
49-*B. oceanisediminis*	467	0.55
37-*B. oceanisediminis*	480	0.55
10-*B. wiedmannii*	483	0.53
18-*B. wiedmannii*	477	0.53
C2-*B. wiedmannii*	476	0.53
2-*B. megaterium*	475	0.52
8-*B. megaterium*	477	0.52
40-*L. fusiformis*	479	0.53
41-*L. fusiformis*	480	0.53
50-*L. macroides*	475	0.53
52-*L. macroides*	468	0.53
48-*L. pakistanensis*	481	0.52
19-*B. aryabhattai*	474	0.53
20-*B. aryabhattai*	474	0.53
43-*B. niacini*	473	0.55
5-*B. niacini*	476	0.55
4-*B. drentensis*	481	0.55
31-*B. drentensis*	466	0.56
22-*B. simplex*	474	0.54
28-*B. simplex*	470	0.52
44-*B. firmus*	478	0.55
45-*B. thioparans*	476	0.54
1-*B. onubensis*	500	0.52
33-*O. longus*	499	0.52
C1-*B. toyonensis*	471	0.54
9-*B. persicus*	474	0.56

Polymorphism and Genetic Diversity among isolated bacillus strains: All 39 isolates were aligned using both Clustalx and Maft build in ape package (Paradis et al., 2004). As the alignment results of the two software were found to be identical, the robustness of the alignment method is ensured. General information about the polymorphisms on the isolates is found in a table (4). The number of sites was 442, of which 233 sites were fond monomorphic, 209 sites were polymorphic. These polymorphic sites divided into 104 parsimony informative sites (i.e. sites that have a minimum of two nucleotides that are present at least twice) and 105 singleton variable sites (non- informative). In the present data set number of haplotypes were 22 where haplotype diversity was 0.94±0.03 Nucleotide diversity (π) is defined as the average number of nucleotide differences per site between two sequences Nei (1987). Estimate of nucleotide diversity was found to be 0.10, where the average number of nucleotide differences was 44 (table 5).

Figure 4: Base frequencies for all the 39 isolates bacillus isolated strains

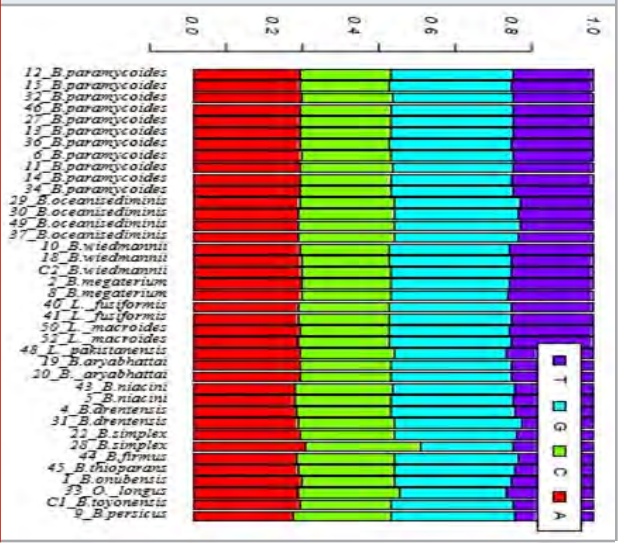


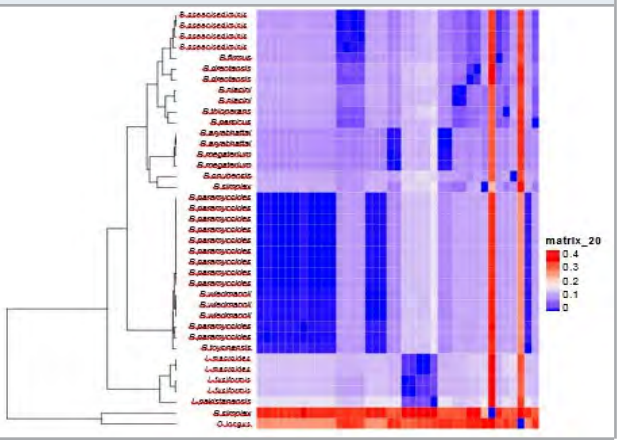
Table 4: Length of conserved regions, conservation, homzigosity and P-valuesof 39 isolates

Region	Start-End Conservation	Homozigosity	P-value
61-138	0.61	0.1	0.04
63-140	0.61	0.9	0.04
65-157	0.60	0.9	0.02
82-160	0.61	0.9	0.03
209-290	0.60	0.95	0.04
244-344	0.60	0.97	0.02
271-393	0.60	0.97	0.01

Table (6) shows the conserved regions along the 39 isolates and measurements of conservation (C), homosigoisty and P- value. Conservation (C) is calculated as the proportion of conserved sites in the alignment region, where homosigosity is measured as 1- heterzygosity. A total of seven conserved regions were observed, 6 of which are overlapping. The P-value for 6 of these seven con- served region were 0.05, where the P-value for the seventh conserved region was0.01 16S rRNA gene sequencing has been a widely used method for the identification of bacterial isolates from different environmental samples. The traditional culture methods for identification depending on phenotypic characteristics and biochemical testing. These methods require minimum equipment but

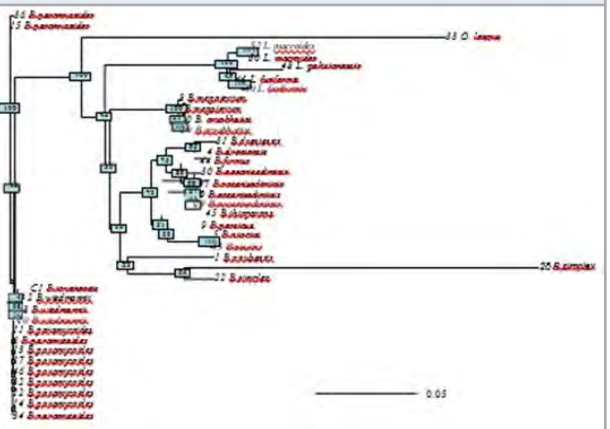
differentiate only between common species (Springer, et al., 2001; Tortoli, et al, 2001). The 16S rRNA gene sequencing has been described and available technique for bacterial identification (Mauti, et al., 2019). The timing needed for 16S rRNA gene sequencing confirmd as speed and accuracy for identification of bacterial isolates comparing to normal traditional identification methods (Cloud, et al., 2002; Kirschner,et al., 2000; Turenne, et al., 2001; Sabir, et al., 2013; Al-Yahyawyet al., 2019).

Figure 5: Cluster analysis of 36 isolated bacillus strains



Comparing of microbial communities with the sequenceswith closely related species in the similar 16s r RNA sequences of databases help all scientists all over the world to identify the bacterial species in

Figure 6: Phylogenetic tree using the Neighbor-Joining method Maximum Likelihood method indicated and reconstructing the evolutionary relationships of isolated bacillus strains.



their samples (Burns, et al., 2015; Claesson, et al., 2010). The 16S rRNA gene become more commonly used for rapid identification of unknown isolates environmental samples and for confirmation of variant sequence data that might have been generated by the higher-throughput methods (Clarridge 2004). The number of 16S rRNA gene sequences in different databases is increased daily (Boudewijns, et al., 2006). Our results confirmed that a description of the isolated species as well as their sequence length and the percentages of GC content of each isolate these data are inagreement with literatures (Turenne, et al., 2001; Sabir, et al., 2013; Al-Yahyawyet al., 2019).

Table 4. Estimated parameters of the polymorphic sites for 39 bacillus isolated strains

Isolate	No. Sites	No. Mono. -morphic sites	No. Poly -morphic sites	Parimony informative	Singleton variable sites
	442	233	209	104	105

Table 5. Estimated parameters of DNA polymorphism for 39 bacillus isolated strain

No. Haplotypes	Haplotype diversity±SD	Nucleotide diversity(π)	Average number of nucleotide
difference	22 44	0.94±0.03	0.10

No noticeable variation in the base frequencies for all the 39 isolates except for B.simplex where this species has a noticeable access in cytosine base. Also obtained results shown all 37 species were clurested together in the second cluster occupying different clades (Dréan,et al., 2015).I n the present data set we show number of nucleotide diversity according to Nei (1987). A total of seven conserved regions were observed along the 39 isolates and measurements of conservation (C), homosigoisty and P- value. Conservation (C) is

calculated as the proportion of conserved sites in the alignment region, where homosigosity is measured as 1- heterzygosity (Paradis,et al., 2004).

Table 6. Length of conserved regions, conservation, homzigosity and P-values of all 39 bacillus isolated strains

Region Start-End	Conservation	Homozigosity	P-value
61-138	0.61	0.1	0.04
63-140	0.61	0.9	0.04
65-157	0.60	0.9	0.02
82-160	0.61	0.9	0.03
209-290	0.60	0.95	0.04
244-344	0.60	0.97	0.02
271-393	0.60	0.97	0.01

CONCLUSION

In conclusion, these preliminary results confirm the fact that species identification by bioinformatics analysis approach may be sufficient for analyzing cultured and uncultured bacterial communities from environmental samplesto species level in some cases. By the public databases it resolve wasting time by traditional bacterial identification methods, but should be confirmed by complementary DNA sequencing. High-quality DNA sequences with SNP analysis could increase the power of databases to facilitate bacterial identification and discrimination between closely related species (Uelze, et al., 2020).

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Detection of two Molecular Identified Strains of Penicillium and Patulin Producing from fresh and Dried Apple in Jeddah, Saudi Arabia

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ABSTRACT

Storage fruit at suboptimal conditions would promote the fungal growth and perhaps the production of mycotoxin which probably transfer to apple products such as apple juice and other apple products, such as purees and baby food is consumed by infants which can be dangerous for human consumption. Therefore, the objective of this study was to investigate a samples of fresh and dry apples including (40) fresh apple and (20) dry apple were collected from different markets in Jeddah governorate, Saudi Arabia. In this investigation, mycoflore from apple samples were isolate the by using Potato Dextrose Agar (PDA) medium at a temperature of 28 C. The results of this study include the isolation and molecular identification of 5 genera and 39 different species which were identified as *Penicillium, Aspergillus, Rhizopus, Alternaria*, and *Fusarium*. The most dominant genera was Penicillium sp. Then we used thin layer chromatography (TLC) to test about 50 Penicillium isolates for their ability to produce patulin. Moreover, *P. expansum* and *P. chrysogenum* were found to be toxigenic fungi with higher probability to produce and secrete patulin. In conclusion, fresh or dried apples may be contaminated by certain strains of fungi that produced toxins, specially patulin which cause may human health problems.

KEY WORDS: MYCOTOXINS, PATULIN, APPLE, TLC, PENICILLIUM, P. EXPANSUM, P. CHRYSOGENUM.

INTRODUCTION

Apples are fruits with high levels of sugars and other nutrients which making it favorable media for fungal growth and production of mycotoxin. Apples can be infected by fungi through a split or other injury of the fruit which breaks the skin of the apple. Then fungi in apart from effects of relative humidity and high temperature can produce some enzymes as pectinase which break down apple pectin then absorb these nutrients (Ruangwises et al., 2013, Ruangwises et al., 2011, Sani et al., 2014). Suboptimal storage conditions of apples would increase fungal growth and mycotoxin production (Singh and Sharma, 2018). Filamentous fungi which occur naturally in fruits produce mycotoxins as secondary products that represent a very large group of different substances and compounds including patulin, ohratoxin , aflatoxins and alternaria toxins that produced by different types of mycotoxigenic species (Henry et al., 2001, Hussain et al., 2008). During the growing season, moulds can infect fruits as apple.

These infections can also happen during harvesting, handling, transportation, storage, marketing conditions and by consumer himself. This infection can affect the fruit itself and can affect the products of this fruit as apple juice (Erten et al., 2019). One of the oldest methods that used for preserving food is drying it producing dried apple

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in this case however, drying the fruits making it very sensitive to change in color due to drying conditions as temperature and oxygen as well as storing conditions of the packed products. Therefore, polymeric materials that used in packing of dried apples should have appropriate barrier characters of preventing microorganisms, water, oxygen, carbon and nitrogen from entering the package. In general, dried fruits is considered less to spoilage by fungi than fresh apple (Hussain et al., 2008).

There are many fungi that can cause apple spoilage, the most common one is *Penicillium expansum* and *Monilinia fructigena* (However, there are other genera of fungi that also can affect the apples and were isolated from apples in previous studies. These genera include *Colletotrichum, Xylaria*, *Botryosphaeria* were found in the study of Kamkar et al. (2005). Another fungi genera called *Rhizopusoryzae* as found in the study of Rouissi (2012) and *Aspergillus spp*. which is isolated from apples and know for causing fruit infections and cause allergies in human (Ruangwises et al., 2013). Other studies indicated *Cladosporium spp*. to be an infection species as they found them in stored apples, besides *Penicillium, Aureobasidium, Sporobolomyces, Cryptococcus* and *Alternaria spp*. (De Melo et al., 2012).

In general, it was found that there are more than 90 species of fungi that can be a causative agent to the infection of apple causing apple decay during storage. *Penicillium spp*., including *Penicillium expansum* when infect apple cause production of blue mold which is soft rot on the apple and they are considered that most commonly reported infectious agents. Other species that can cause decay of apple during storage include *P. viridicatum*, *P. brevicompactum, P. solitum* and *P. expansum* which found to produce patulin in apples. Patulin (PAT) is a mutagenic, immunotoxin and neurotoxic mycotoxin that produced by some types of fungi. Therefore, it is very important to detect and ensure the free of apple fruit from P. expansumespecially when these apples are used for juice production (Shim et al., 2004).

Penicillium spp. is a large group that contained at least 150 species, similar in morphology however, there is a great group of variability among many species of them therefore, and we can recognize more than 1,000 different phenotypes. This inherent variability is very important in identifying the type of fungi; however, only 70 to 80 % of the isolate species even when isolated from common sources are readily identifiable. Morphological criteria and characters of *Penicillium spp*. are the most taxonomic keys to identify *Penicillium spp*. Therefore, *Penicillium spp*. can be identifying upon both of micro and macro morphology besides produced color in the mycelium or when diffused into the growth medium. Moreover, molecular techniques are also used to identify this type of fungi as in recent studies; it is found that they are genotypic characterizations of *Penicillium spp*. that found to be useful in identifying it.

Several methods for detecting DNA polymorphism in fungi have been used to detect intraspecific and interspecific variation in *Penicillium sp* and other genera (Sebage, 2004, Novotný et al., 2019). Therefore, many isolates were collected and initially characterized using phenotypic and physiological parameters. Moreover, Molecular techniques have been used for the detection of *Penicillium sp* from isolated samples (Sebage, 2004, Shamsi et al., 2016).

MATERIAL AND METHODS

Apples samples collection: Sixty samples which include 40 samples of fresh apple (Red Delicious apple and Granny Smith apple), 20 samples of dried apple were collected from different markets of Jeddah governorate, Saudi Arabia in August 2018. Samples were collected in sterilized polyethylene bags, transferred into laboratory and preserved in refrigerator until the study time. Isolation fungi from fresh and dried apple using smear method: Swap from each sample of apple was prepared to inoculate agar plates. The plates were incubated at 25^{0}C for 7 day. PDA medium was used as a subculture medium to identify the isolates.

Direct plating method for dried apple: Each dried apple sample was cut aseptically into small pieces. Three pieces were transferred to the surface of agar plate containing Potato Dextrose Agar medium (PDA) from OXOID, UK. The plates were incubated at 25°C for 7 days (Pitt et al., 1992). PDA medium was used as a subculture medium to identify the isolates.

Morphological and molecular identification of fungal isolation

Diversity analysis: Culture and morphological characteristics of the isolated fungi of 5-10 days, grown on PDA were assessed by examination under a light microscope. Subsequently, slide preparation of fungi taken from the culture were stained with drop of Lacto phenol cotton blue and examined for spores and mycelia features using light microscope (x 400). Identification of fungi was based on comparison of morphology, color, shapes of spores and colony characters as descript by Domsch et al. (2007) and Watanabe (2010).

Genotypic Identification of two strains isolates: Two strains isolates (*P. expansum* and *P. chrysogenum*) were injected in (100) ml of Erlenmeyer flasks containing 20 ml potatoes dextrose broth (PDB) then incubated at 28 °C for 5 days, then was filtration of mycelia using sterilized filters. The fungal mycelium was ground to a powder by sterilized pestle and mortar using liquid nitrogen. Powders were transferred to 1.5 ml Eppendorf tube and stored at -20°C. Thirty mg of frozen mycelium powder were resuspended and lysed in 500 µl of lysis buffer (pH7•8) and incubated in a water bath at 37°C for 60 mins. The lysis buffer was a mixture of 20 mmol/l sodium acetate, 40 mmol/l Tris-acetate, 1 mmol/l EDTA and 1% SDS. Extraction of the DNA was done by Gene JET Genomic DNA extraction kit (Thermo Scientific, USA) and the PCR technique was used to magnify internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using two primers; ITS1(CTTGGTCATTTAGGGAAGTAA)

and ITS4(TCCTCCGCTTATTGATATG) in a thermal cycler (Esco health care, Swift max pro, Malaysia). The reaction mixture (50 µl) included 5 µl each of the primers, 3 µl of templet DNA, 50 µl of nuclease-free water and 25 µl of green PCR mix (Promega, Go Taq ® Green Master Mix, USA).

DNA visualization: On to 1.5% agarose gel, the PCR products were tested and run for 45 mines at 130 volts electrophoretic gel, ethidium bromide was using as stained and visualized under UV light. To quantify and identify PCR products was used the DNA marker. Then the samples were sent to Macrogen company, South Korea for purification and sequencing .

DNA sequencing: Using Big Dye terminator cycle sequencing kit (PE-Applied Biosystems, USA) for PCR product from two fungal strain. Using BLAST Genbank general databases from National Center for Biotechnology information (NCBI) database for sequence identities and used Jukes-Cantor model to construct neighbor-joining tree as described by (Najjar et al., 2019).The sequencing data were submitted to GenBank and the obtained accession number was recorded for tow fungal strain.

Table 1. Total colony (TC per g), number of appearance out of 40 samples of fresh apple and frequency occurrence (FC)of fungal species from samples at 28C for 7-10 days on PDA cultural medium.

Fungal species	Total count (TC)	Total count% (TC %)	Frequency of occurrence in 60 samples (FC)	Frequency % (FC%)
Penicillium sp	1716	64.7	84	44.2
P. expansum	618	23.3	30	15.8
P. chrysogenum	186	7.0	21	11.1
Alternaria sp.	38	1.4	12	6.3
Aspergillus sp.	32	1.2	10	5.3
Aspergillus flavus	11	0.5	5	2.5
Aspergillus niger	21	0.8	14	7.4
Rhizopus sp.	8	0.3	3	1.6
Fusarium sp.	21	0.8	11	5.8
Total counts per sample	2651	100	190	100

Table 2. Total colony (TC per g), number of appearance out of 20 samples of dried apple and frequency occurrence (FC) of fungal species from samples at 28C for 7-10 days on PDA cultural medium.

Fungal species	Total count (TC)	Total count (TC) %	Frequency of occurrence in 60 samples (FC)	Frequency % (FC)
Penicillium sp.	27	49.1	84	44.2
P. expansum	8	14.5	30	15.8
P. chrysogenum	1	1.8	21	11.1
Alternaria sp.	3	5.5	12	6.3
Aspergillus sp.	7	12.7	10	5.3
A.niger	7	12.7	14	7.4
Fusarium sp.	2	3.7	11	5.8
Total counts per sample	55	100	190	100

Screening of fungi for mycotoxins production

Growth of fungal isolates: All fungal isolated were plated in duplicate on PDA to test their ability to produce mycotoxins according to Samson and Hoekstra (1988). The center of each plate was inoculated with a plug cut from a plate culture using a flame-sterilized cork-borer (4 mm internal diameter) and the plates were incubated at 25°C for 10 days. The diameter of the fungal colony (mm) was determined.

Preparation and screening of the fungal extracts: All fungi isolated were tested by a rapid screening method for mycotoxin production (Filternborg and Frisvad, 1980). From 10-day old plate culture, 5 agar discs were

cut out near the center of the colony with a flame-sterilized stainless steel cork-borer (4 mm) and removed using a flame- sterilized scalpel. Five discs of growth were removed and extracted by mixture of chloroform: methanol (2/1 V/V). The organic layer were collected in small begoes and preserved at 20°C until used.

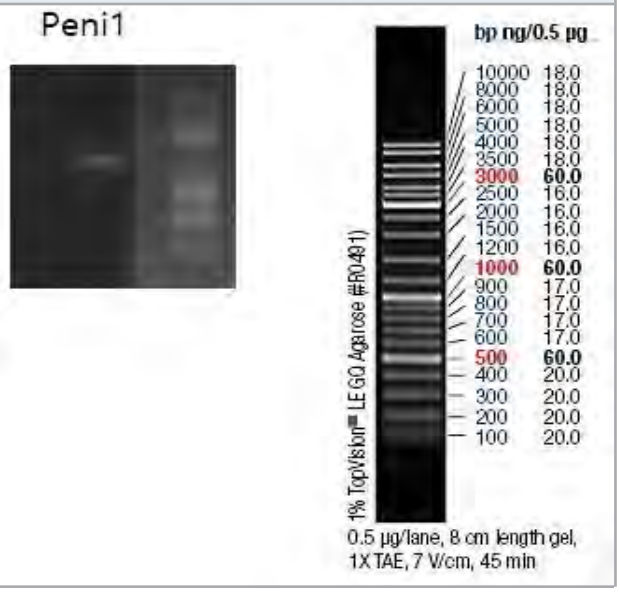
Table 3. Molecular identification of fungal strains recovered in the present study, their GenBank accession No. and % identity with closely related strains

Fungal species	Fungal strains isolated in the current study		Closely related strains accessed from GenBank			
	Strain No.	Accession No.	Fungal species	GenBank No.	Coverage (%)	Identity (%)
P. expansum	RZ1	GU561988.1	*P. expansum*	GU561988.1	100%	100%
P. chrysogenum	RZ2	KT601570.1	*P. chrysogenum*	KT601570.1	91%	99.94%

Table 4. Screening of two selected isolates for the production of mycotoxins by TLC

Type of apple	Toxin	Tested Fungi	Production of Patulin	Characteristics
Fresh apple	Patulin	*Penicillium sp.* (20) *P. chrysogenum* (1) *P. expansum* (1)	- + +	Yellow fluorescence after exposure to 0.5% of 3-methyl-2 benzo-thiazolinone hydrazine hydrochloride (MBTH) spray followed by 15 min at 110C.
Dried apple	Patulin	*Penicillium sp* (8) *P. chrysogenum* (1) *P. expansum* (1)	- - -	
+ : Present, - : Absent				

Figure 1: Gel electrophoresis of PCR product from *P. chrysogenum* strains. Data shown was obtained with ITS1/4 primer.



Detection of patulin-producing strains by thin layer chromatography (TLC): RF was used as standards for the toxins produced by the tested fungi (patulin).

Preparation and development of TLC plates: For routine examination of extracts, aluminum-backed, DC-Alufolien- Kieselgel 25 plates, silica gel matrix, with fluorescent indictor at 254 nm, were cut to 10 x 10 cm and spotted along a line 1.5 cm from the bottom with 10 µl aliquots of extracts or standards. The plates were developed in the solvent system TEF :Toluene, Ethyl acetate 90% and Formic acid (5:4:1 v/v) at the room temperature until the solvent front reached a line marked 1 cm from the top of the plate(Roberts and Patterson, 1975, Bokhari, 1993). After development, the plates were removed from the solvent, and air-dried in a fume cabinet. The plates are sprayed and dried at 100 °C in a hot-air oven, then examined in a Chromato-Vue cabinet (Model UVP Upland, CA USA) under visible light or short wavelength UV light (254 nm) and long wavelength (366 nm) as described by Samson et al., (2000).

Detection and identification of mycotoxin on TLC plates: Development chromatograms were examined under visible light for colored substances, under UV light for fluorescent substance with and without the use of spray reagent. The detection system for particular toxin is shown Table 1. The patulin gives a fluoresced yellow color after exposing the Chromatography plate for 0.5% of 3-methyl-2benzo-thiazolinone hydrazine hydrochloride (MBTH) spray followed by 15 min at 110 ^{0}C. fluorescent color, and ochratoxin gives a fluoresced

blue color after exposing the Chromatography plate for Ammonia vapor for 10 min and heating at 110°C for 5 min.

Statistical analysis: The means of variable ± SD were recorded and all data was subjected to statistical analysis using SPSS 16, and the differences between mean values as determined by Student's t-test were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

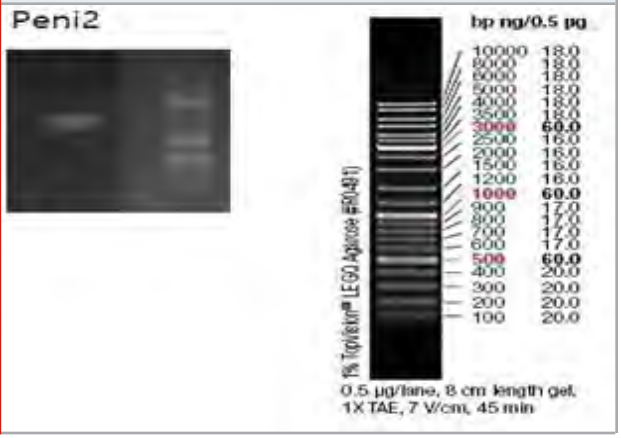
This investigation involve an inclusive survey of the mycoflora associated with apple fruits in 60 samples collected from different markets in Jeddah governorate, Saudi Arabia. The tested samples embrace fresh and dry apple (40 samples for fresh apple and 20 samples for dried apple). In this respect, Akinmusire (2011) and Chukwuka et al. (2010) mentioned that wide range of microorganisms can affected fruits such as fungi which have a severe menace to production of fruits. Spoilage impute to any change in the condition of food making it less agreeable, or even toxic. Furthermore, presence of mycotoxin in fruits is an ongoing global concern. Mycotoxin contamination is considered an unavoidable and unpredictable problem, even where perfect agricultural, storage, and processing practices are implemented, posing a difficult challenge to fruits safety (Patriarca, 2019). Additionally, during food processing many mycotoxins are not easily eliminated because of their stability against heat, chemical, and physical treatments (Barkai-Golan, 2001, Battacone et al., 2005, Baert et al., 2007, Bilandzic et al., 2010, Basson et al., 2019).

Fruits contamination can also pose an extra hazard for food safety due to the possible relocate of mycotoxins to fruits products such as juices and other products, such as purees and baby food is consumed by infants which can be dangerous for human consumption. Therefore, there is a growing and continuous interest in the study of the basic biology and genetics of toxigenic *Penicillia* because of their natural occurrence in fruit and fruit products and of the toxic effects of their secondary metabolites on humans (Puel et al., 2010, Frizzell et al., 2014; Patriarca et al., 2019). PAT is the most mycotoxin found in apples and in their products which discompose a serious risk to the health of consumers. Its presence is related primarily to contamination by *P. expansum* in apples post-harvest (Paterson et al., 2006; Pianzzolla et al., 2004). Acute PAT exposure may lead to vomiting, nausea and other gastrointestinal symptoms such as intestinal haemorrhages, gastric ulcers, and lesions in the duodenum, as well as alterations in the intestinal barrier function accompanied with kidney damage (Speijers et al., 1988, Mahfoud et al.,2002, De Melo et al., 2012). Arnau et al., (2019) have reported that these alterations were observed in rats after a dose of 1 mg/kg bw in rats.

During the first part of this investigation, it was isolate 39 species belonging to 5 fungal genera which were identified as *Penicillium, Aspergillus, Rhizopus, Alternaria*, and *Fusarium* (Table 1, 2). The findings of this study showed that, *Penicillium* was the genus most frequently isolated from the fresh and dried apple. Among the 20 species of *Penicillium, P. chrysogenumr* and *P. expansum* were the most common species and was identified using molecular identification (Table 3) wherefore, dried apples was less contaminated by fungi, since dried apple contain very low moisture content compared to fresh apples, which make them less exposed infection by fungi. However, fruit drying is one of the oldest methods for preserving Fruits for centuries. Fruit may be dried as whole fruit e.g., grapes, in sliced form e.g., banana, mango, papaya, kiwi, apple etc.), in puree form e.g., mango, apricot (Ratti and Mujumdar, 2004). The drying of fruits allows for their better preservation by reducing water content, thus inhibiting microbial growth and enzymatic modifications.

These products are thought to be resistant to microbial spoilage because of their low water activity, high acidity and sugar content, as a consequence of drying process. In a recent study, Chalupowicz et al.,(2020) have reported that Penicillium appeared to be the most fungal genus infected apple. In addition, Barkai-Golan (2001) and Paola et al., (2008) reported that many Penicillium species were the causative agent of post-harvest diseases in which they infect a wide range of crop. DovPrusky et al., (2010) found that *P. expansum* responsible for rotting apple fruits, in which it lead pH to decreases from 3.95 to 4.31 in the healthy mesocarp to values ranging from 3.64 to 3.88 in the rotting tissue. The growth rate and PAT production by *P. expansum* are also largely influenced by environmental and endogenous factors of the substrate (Baert et al., 2007).

Figure 2: Gel electrophoresis of PCR product from P. expansum strains (Data shown was obtained with ITS1/4 primer).



Since blue mould is a problem principally occurring during storage, temperature and atmosphere composition are the most important factors determining mould growth and mycotoxin production. Apples are usually stored under controlled conditions: low temperature (0.5–3.5°C) in combination with reduced O2 (1–3%) and elevated CO_2 (0.8–3%) (McCallum et al., 2002). PAT production

has been observed at all temperatures allowing P. expansum and *P. chrysogenum* growth, encompassing an approximate range of 4–30^{0}C (Sommer et al., 1974).

Additional reports on fungi isolated from fresh and dried apple were reviewed by many authors (Sani et al., 2014, Shamsi et al., 2016, Lee et al., 2017, Basson et al., 2019). In Riyadh, Alwakeel (2013) isolated the fungi associated with apple spoilage which included *P. chrysogenum, P. adametzii, P. steckii,* and *Aspergillus oryzae*. The authors demonstrated the *P. chrysogenum* was the most frequent isolate.During the second part of this investigation, we were tested *Penicillium* isolates for ability to produce PAT in conditions in vitro using TLC method (thin-layer chromatography) (Table 4). TLC is a chromatography technique used to separate non-volatile mixtures (Harry et al., 1989).

Figure 3: Dendrogram showing phylogenetic analysis based on the ITS region and NCBI GenBank database for P. chrysogenum strains (Sample name :Penicillin1_ contig_1).

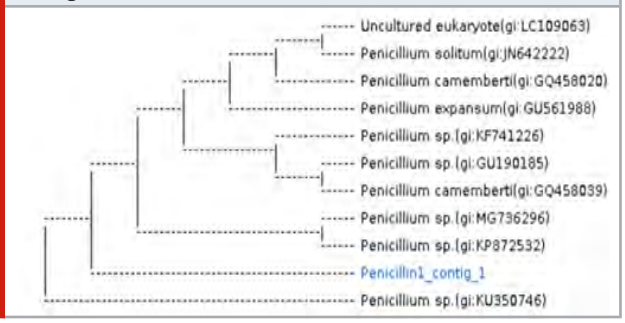
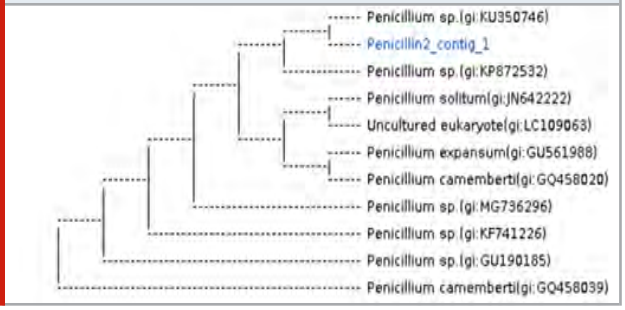


Figure 4: Dendrogram showing phylogenetic analysis based on the ITS region and NCBI GenBank database for P. expansum species (Sample name: *Penicillin*2_contig_1).



The capacity of the two tested strains (*P. expansum* and *P. chrysogenum*) were producing PAT in conditions in vitro, the results were determined by the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase. (The mobile phase must not be allowed to reach the end of the stationary phase.) This ratio is called the retardation factor (Rf). In general, a substance whose structure resembles the stationary phase will have low Rf, while one that has a similar structure to the mobile phase will have high retardation factor. Retardation factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. For this reason, chemists usually apply a sample of a known compound to the sheet before running the experiment.

CONCLUSION

For human safety, food laws should be set up in Saudi Arabia for the occurrence of toxigenic fungi and mycotoxins, in fresh and dried apple even though the dried apple are exposed to heat before consumption due to the heat stability of mycotoxins.

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Evaluation of the Possible Immunomodulatory and Anti-Inflammatory Effects of Gamma-Irradiated Basil, *Ocimum basilicum* Against Arsenic Toxicity in Rats

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ABSTRACT

In view of the widespread incidence of arsenic poisoning around the world, it was necessary to study this phenomenon and analyze it to find out how to treat it through the application of alternative medicine. Gamma irradiation as a phytosanitary treatment of food and herbal materials is increasingly recognized throughout the world by improving their hygienic quality. The aim of this study was to evaluate the therapeutic effect of raw or irradiated basil on rats exposed to arsenic toxicity. Basil was irradiated by gamma rays at dose 10 KGy. Forty-eight adult Wistar albino rats were divided into six groups as follows group-1: Control group, group-2: received 400 mg/ kg of aqueous extract of basil, group-3: received 400 mg/ kg of aqueous extract of irradiated basil, group-4: received 10 mg/kg of sodium arsenate solution, group- 5: received 10 mg/kg of sodium arsenate solution and 400mg/ kg of aqueous extract of basil, and group- 6: received 10 mg/kg of sodium arsenate solution and 400mg/ kg of aqueous extract of irradiated basil. At the end of the experiment (5 weeks), the rats were sacrificed, blood and brain tissue samples were subjected to estimate of the following: CBC, inflammatory markers (CRP, TNF-α, and IL6), immunoglobulin markers (IgA, IgG, IgM), and the levels of oxidative stress and antioxidant in brain tissue (MDA, CAT, SOD, and GSH). The results showed a rise in the antioxidant of basil after the irradiation process. The arsenic caused a significant decreased in levels of HB, RBC, LYM, NEU and PLT and increased levels of WBC and reticulocyte count as compared to the control group. Also, the rats exposed to arsenic showed a significant increase in inflammatory markers in serum, and oxidative stress in brain accompanied with significant decreased in immunoglobulin markers in serum and antioxidant in the brain. In contrast, the administration of raw basil or irradiated basil extract with arsenic was a helpful factor in alleviation of these side effects. In conclusion, our findings showed that the irradiation process enhanced antioxidants in basil plants which attenuated arsenic toxicity.

KEY WORDS: BASIL; GAMMA IRRADIATION; ARSENIC; HEMATOLOGY; INFLAMMATION; IMMUNE SYSTEM; BRAIN.

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INTRODUCTION

Arsenic is a toxic substance that occurs naturally and is found in water, rock soil and many foods (Duker et al., 2018).Arsenic toxicity affects millions of people in different parts of the world through the ingestion of arsenic-contaminated drinking water and food (Huq et al., 2006; Flanagan et al., 2012).In recent years, arsenic toxicity has created significant public concern (Duker et al., 2018).It has no taste or smell, which makes it particularly hazardous, so one can be exposed to it without knowing it (ATSDR, 2017). The increasing presence of variable amounts of arsenic in the environment presents major risks, as exposure through inhalation, ingestion and dermal contact can cause various adverse effects on health systems (Vimercati et al., 2017, Chiocchetti et al., 2018 Mochizuki et al., 2019, Tutkun et al., 2019), (Zhao et al., 2019).

The National Center for CAM (complementary and alternative medicine) was reported as a category of healthcare and medical systems (NCCIH, 2018).A systemic review conducted by Eardley et al. (2012) found that, for many reasons, people are using CAM, including its availability, perceived health, and disease prevention. As one of the main forms of CAM is herbal medicines, which uses parts of a plant or whole plants to avoid and cure diseases (Bent, 2008; Pan et al., 2014).The herbal medicinal products are characterized by the presence of complex chemical compounds responsible for the pharmacological activities that contribute to health benefits(Bent, 2008).Basil plants considered from herbal medicine that has eminence value as a cure for various diseases (Patel et al., 2018).

Basil (*Ocimum basilicum* L.) is an herbaceous and aromatic plant worldwide cultivated which belongs to the *Lamiaceae* family (Jakovljevic et al., 2016). It has several therapeutic properties including antioxidant, anti-aging, anticancer, antiviral, antimicrobial, anti-genotoxic, and anti-inflammatory(Sakr and Al-Amoudi, 2012; Shirazi et al., 2014). Studies have shown many pharmacological effects for basil in several diseases such as liver fibrosis(Alomar and Al-Attar, 2019), diabetes mellitus (Widjaja and Rusdiana, 2019), asthma (Eftekhar et al., 2019), anemia (Zangeneh et al., 2019), and cerebral injury (Singh, Krishan and Shri, 2018).Based on the pharmacological and therapeutic properties, basil has played an important role in both traditional pharmaceutical products and contemporary pharmacological and clinical science(Shirazi et al., 2014). But there may be concerns about the use of herbs, which is that herbs are susceptible often to contamination during processing or storage by micro-organisms and insect pests. This leads to shortens their shelf life and triggering serious illness in some cases, particularly if the herbs contaminated with *Salmonella* and *Staphylococcus aureus* (Chatterjee et al., 2016). Thus, herbs should be subjected to sterilization or microbial treatment before use.

There are various techniques for the decontamination of medicinal plants, including irradiation (Garg and Gupta, 2016).This is a physical process that applies high-energy ionizing radiation to the plants in order to enhance their safety and shelf-life (Byun et al., 1999; SádECká, 2007; Alothman, Bhat and Karim, 2009). In particular, gamma irradiation seemed to be the best way to decontaminate herbs from microbes without triggering quality changes(Lee et al., 2005). As the absorbed energy of radiation can break the bonds of DNA molecules in microorganisms present in the product and inactivates certain enzymes, this greatly reduced its damaging impact on products. Not only microorganisms are destroyed, but even gametes, insects and parasites are prevented from reproducing, resulting in various preservative effects, (Farkas, 2006).

Moreover, irradiation serves as a safe technique in food processing supported by many internationally recognized organizations, where joint (Food and Agriculture Organization/ International Atomic Energy Agency/ World Health Organization)Expert Committee on the Wholesomeness of Irradiation of Food has ruled that food subject to low irradiation dosage (up to 10 kGy) is safe and not need any testing of toxicology (Wen et al., 2006). The present study was therefore designed to evaluate the possible immunomodulatory and anti-inflammatory effects of raw and irradiated basil (*Ocimum basilicum*) against arsenic toxicity in rats.

MATERIAL AND METHODS

Chemicals and kits: Sodium arsenate was obtained from British Drug Houses (BDH) chemical company, England, UK. Diethyl ether was obtained from Sigma-Aldrich company, Louis, USA. Kits for assays of immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), tumor necrosis factor-alpha (TNFα), interleukin (IL-6), C- reactive protein (CRP), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and malondialdehyde (MDA) were obtained from Abcam Chemical Company, Cambridge, UK. kits for assay Total capacity antioxidant were obtained from Cell Biolabs company, California, USA. Kits assay protein concentration in tissues (Pierce TM BCA Protein Kit) was obtained from Thermo Fisher Scientific Company, Waltham, US.

Plant material and preparation of extract: The basil (*Ocimum basilicum*) leaves were purchased from the local traditional market in Jeddah, Saudi Arabia. The water extracts of raw or irradiated dried basil leaves were prepared according to the method described by Ghazwani et al. (2020). The total antioxidant capacity was measured in basil extracts using the Total Capacity Assay kit with CAT# STA-360.

Gamma Irradiation treatment: The samples of dry basil powder were irradiated with 10 KGy of gamma rays after the leaves were transferred into polyethylene bags, using a Cobalt-60 source at a dose rate of 4.75 KGy/h at the National Centre for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt.

Experimental animals and Design: The study was carried out using adult female Wistar rats weighing (150-200g); they were obtained from faculty of pharmacy at King Abdulaziz University. The animals were housed in cages and received normal rat chow and tap water in a constant environment (room temperature 28 ± 2°C, room humidity 60±5%) in a 12 h light and 12 h dark cycle. Rats were kept under supervision for two weeks before the experiments started and during all stages of the whole experiment. Animal's procedures were performed in accordance with the Ethics Committee of the King Fahad Medical Research Center and in accordance with the recommendations for the proper care and use of laboratory animals. In the experiment, 48 rats were divided into six groups each of 8 rats as follows:

Group 1: normal control rats were given only distilled water. Group 2: rats received 400 mg/ kg of aqueous extract of raw basil (Ezeani et al., 2017). Group 3: rats received 400 mg/ kg of aqueous extract of irradiated basil (Ezeani et al., 2017). Group 4: rats received 10 mg/kg of sodium arsenate solution (Firdaus et al., 2018).Group 5: rats received 10 mg/kg of sodium arsenate solution and 400mg/ kg of aqueous extract of basil. Group 6: rats received 10 mg/kg of sodium arsenate solution and 400mg/ kg of aqueous extract of irradiated basil. All doses were given through an oral gastric tube daily for five weeks. At the end of the experiment, rats fasted overnight for scarification.

Blood samples were withdrawn by a heparinized capillary tube from the retro-orbital plexus of each rat under anesthesia with diethyl ether, it is put into two tubes, one is ethylenediamine tetra-acetic acid (EDTA) tube and the other is a serum-separating tube. The EDTA tube immediately turned to lab analysis while serum-separating tube centrifuged at 3000 rpm for 15 min to separate serum and then stored at -40° C until the biochemical analysis is done. Directly after preparing the blood sample, rats sacrificed and the brain was kept in ice for homogenate preparation.

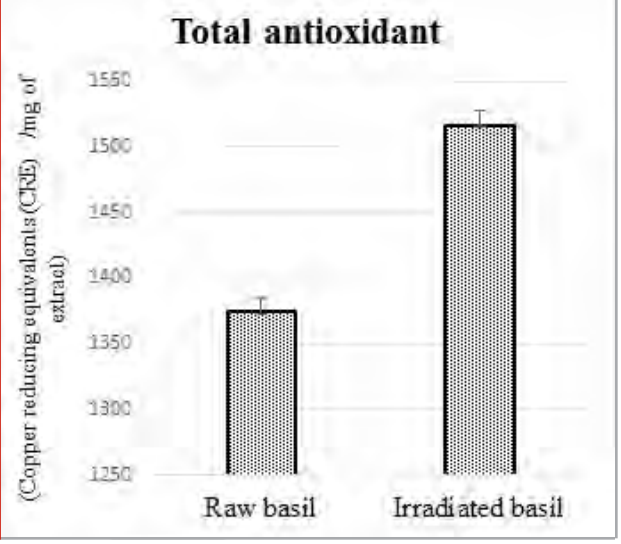
Biochemical analysis: The biochemical analysis for complete blood count (CBC) was done in Kingdoms Labs. The blood serum IgG, IgM and IgA, TNFα, IL-6 and CRP were identified using the kits with CAT# ab189578, ab157735, ab157738, ab46070, ab119548, and ab108827, respectively. For tissue analysis, the brain was homogenized and estimated each of SOD, GSH, CAT and MDA according to the kits with CAT# ab65354, ab138881, ab83464, and ab118970, respectively, while the protein concentration estimated in the brain was used Kits with CAT# 23225.

Statistical analysis: The data of each group were analyzed using MegaStat 9.4 (add-in for Excel). The data were expressed as arithmetic mean and standard deviation of the mean (SD).Differences between groups were analyzed for parametric parameters using one-way variance analysis (ANOVA), the least significant difference equation (LSD). A P value below or equal to 0.05 was considered significant.

RESULTS AND DISCUSSION

Antioxidant in irradiated plant leaves: The effect of irradiation on antioxidants is showed in figure 1. Where leaves treated with gamma irradiation (10kGy) showed a change, a 10.3% increase in the content of total antioxidants compared to raw leaves not treated with radiation.

Figure 1: Total antioxidants in raw and irradiated basil



Complete blood count (CBC): The effects of aqueous extracts of raw basil or irradiated basil for 5 weeks on the levels of hemoglobin (HB), red blood cells(RBC), hematocrit (HCT), platelets (PLT), White blood cells (WBC), WBC differential and reticulocyte count in rats exposed to arsenic are present in Tables 1 and 2. No effect of raw or irradiated basil administration in normal animals on all previous parameters was noted. Rats, which were exposed to arsenic showed a significant decrease in levels of HB, RBC, PLT, neutrophils (NEU), and lymphocytes (LYM) compared to the control group. While there was a marked increase in the levels of HCT, WBC, monocytes (MON) and reticulocyte count compared to the control group. Administration of raw and irradiated basil along with arsenic significantly reduced arsenic toxicity effect by the improvement levels of these parameters.

Antibodies: The extracts of raw and irradiated basil had distinct effects on antibodies in rats exposed to arsenic that are showed in Table 3. The treatment with raw or irradiated basil to normal animals not producing an effect on levels of serum IgG, IgM and IgA. The arsenic group showed a significant decrease in IgG, IgM and IgA levels compared to the control group. The treatment with basil extract as raw or irradiated along with arsenic dose was showed a significant increase in those antibodies' levels, compared with the group of rats given only arsenic.

Inflammatory markers: The level of markers of inflammation in rats exposed to arsenic which received raw or irradiated basil as a treatment are showed in Table 4. In normal animals, the administration of raw

or irradiated basil has not had an effect on the levels of serum TNF, IL-6 and CPR. The rats exposed to arsenic showed a significant increase in levels of TNF, IL-6, and CPR as compared to the control group. Giving raw or irradiated basil extract significantly reduced inflammatory markers induced by arsenic.

Table 1. Effect of basil extracts on hematological parameters of rats exposed to arsenic

Test / Group	HB (g/dl)	RBC (10^6/mL)	HCT (%)	PLT (10^3/mL)
C	12.661 ±1.258	4.450± 0.575	29.635± 1.870	263.13± 52.58
B	12.725 ± 0.902 bbb	4.570± 0.491 bbb	29.286± 0.516 bbb	274.50± 19.17 bbb
IB	12.810 ± 0.756 bbb	4.568 ± 0.487 bbb	29.518± 0.908 bbb	277.25± 13.63 bbb
A	8.748 ± 0.590 aaa	3.100 ± 0.524 aaa	39.058± 0.655 aaa	143.25± 7.96 aaa
B + A	10.918 ± 0.71 aaa bbb	4.066 ± 0.463 bbb	30.978± 1.748 bbb	188.38± 10.89 aaa bb
IB + A	11.249 ± 0.290 aabbb	4.151± 0.164 bbb	32.330± 1.863 aaa bbb	177.88± 10.47 aaa bb

Values are the mean of 8 observation ± SD, Significant different from C value at P < 0.05[a], 0.01[aa], 0.001[aaa], Significant different from A at value at P < 0.05[b], 0.01[bb], 0.001[bbb]

Table 2. Effect of basil extracts on hematological parameters of rats exposed to arsenic

Test / Group	WBC (10^ 3 / mL)	WBC differential			Reticulocyte Count (%)
		NEU (%)	LYM (%)	MON (%)	
C	5.661 ± 0.508	62.403 ±1.869	28.16 ±2.658	4.113±0.631	1.413± 0.416
B	5.255 ± 0.532 bbb	63.388 ±1.886 bbb	27.61±4.022 bb	4.250±0.460 bbb	1.100± 0.185 bbb
IB	5.134 ± 0.604 bbb	64.538 ±1.577 bbb	29.46 ±4.530 bbb	4.064±0.261 bbb	1.263± 0.424 bbb
A	9.761± 0.893 aaa	38.051 ±2.361 aaa	20.00 ±2.624 aaa	7.150±0.493 aaa	2.350± 0.540 aaa
B + A	6.160± 0.825 bbb	54.175 ±5.389 aaa bbb	24.95±5.687 b	5.163±0.460 aaa bbb	1.011±0.110 bbb
IB + A	5.978± 0.610 bbb	51.850±5.011 aaa	25.73±5.035 b	5.113±0.500 bbb aaa	1.163±0.518 bbb bbb

Values are the mean of 8 observation ± SD, Significant different from C value at P < 0.05[a], 0.01[aa], 0.001[aaa], Significant different from A at value at P < 0.05[b], 0.01[bb], 0.001[bbb]

Antioxidants and oxidative damage: The results of determining the content of SOD, GSH, CAT and MDA in rats given arsenic along with raw basil or irradiated basil were shown in Table 5.The normal rats treated with irradiated basil showed an increase in the level of SOD than rats treated with raw basil. The level of GSH was

showed an increase in normal rats of both of raw basil group and irradiated basil group as compared to the control group. Arsenic-exposed rats showed a significant increase in MDA levels accompanied by a significant decrease in SOD, GSH and CAT compared to a control group. The management of raw or irradiated basil along with arsenic alleviated the effects of arsenic and resulted in a significantly decreased MDA with significantly increased SOD, GSH, CAT.

Table 3. Effect of basil extracts on immunoglobulins of rats exposed to arsenic

Test / Group	IgG (µg/ml)	IgM (µg/ml)	IgA (µg/ml)
C	517.16 ± 35.137	0.628 ± 0.084	90.180 ± 6.688
B	522.490 ± 41.527 bbb	0.640 ± 0.053 bbb	93.236 ± 7.697 bbb
IB	514.663 ± 32.665 bbb	0.655 ± 0.064 bbb	94.614 ± 5.087 bbb
A	202.500 ± 40.818 aaa	0.226 ± 0.024 aaa	45.779 ± 5.816 aaa
B + A	298.911 ± 32.731 aaa bbb	0.389 ± 0.015 aaa bbb	72.754 ± 4.942 aaa bbb
IB + A	287.915 ± 35.128 aaa bbb	0.408 ± 0.021 aaa bbb	76.745 ± 4.229 aaa bbb

Values are the mean of 8 observation ± SD, Significant different from C value at P < 0.05[a], 0.01[aa], 0.001[aaa], Significant different from A at value at P < 0.05[b], 0.01bb, 0.001[bbb]

Table 4. Effect of basil extracts on the inflammation markers of rats exposed to arsenic

Test / Group	TNFα (pg/ml)	IL-6 (pg/ml)	CRP (ng/ml)
C	17.829 ± 1.221	21.048 ± 2.134	0.134 ± 0.039
B	16.144 ± 1.428 bbb	20.753 ± 1.962 bbb	0.134± 0.034 bbb
IB	16.123 ± 1.216 bbb	20.456 ± 2.940 bbb	0.140± 0.018 bbb
A	40.043 ± 2.453 aaa	35.334 ± 3.973 aaa	0.990± 0.076 aaa
B + A	27.656 ± 2.173 aaabbb	27.413 ± 3.842 aaabbb	0.660± 0.093 aaabbb
IB + A	28.935 ± 2.634 aaabbb	26.876 ± 1.563 aaabbb	0.490± 0.033 aaabbb

Values are the mean of 8 observation ± SD, Significant different from C value at P < 0.05a, 0.01aa, 0.001aaa, Significant different from A at value at P < 0.05b, 0.01bb, 0.001bbb

The irradiation treatment can increase the content of some phytochemicals and the plant's antioxidant activity, thereby increased biological value (Zevallos-Concha et al., 2016; Pereira et al., 2018).The results indicated that the extract of irradiated basil showed a highly significant increase in total antioxidants as a comparison to raw basil. Similar observations were reported in previous studies on basil and some other plants (Khawory et al., 2020; Osman et al., 2020; Rady et al., 2020).

Table 5. Effect of basil extracts on the antioxidants and oxidative damage in the brain of rats exposed to arsenic

Test / Group	SOD (Inhibition rate %/ protein)	GSH (µM/gram tissue)	CAT (µM/mg of protein)	MDA (µM/mg of mg of protein)
C	105.406 ±7.342	90.743 ± 7.044	0.020 ± 0.003	2.496± 0.451
B	107.548 ±9.384 bbb	96.709 ± 3.420 abbb	0.019 ± 0.004 bbb	2.400± 0.422 bbb
IB	114.655 ±8.165 abbb	95.425 ± 4.426 abbb	0.021 ± 0.004 bbb	2.441± 0.433 bbb
A	77.977 ±10.370 aaa	60.248 ± 2.657 aaa	0.009 ± 0.000 aaa	6.005± 0.126 aaa
B + A	82.049 ±8.953 aaabbb	84.259 ± 4.487 aabbb	0.012 ± 0.001 aaa	3.479± 0.610 aaabbb
IB + A	89.249 ±9.381 aaabbb	82.533 ± 3.153 aabbb	0.014 ± 0.003 aabb	3.165± 0.484 aabbb

Values are the mean of 8 observation ± SD, Significant different from C value at $P < 0.05^{a}$, 0.01^{aa}, 0.001^{aaa}, Significant different from A at value at $P < 0.05^{b}$, 0.01^{bb}, 0.001^{bbb}

Basil contains phenolic compounds and flavonoids (Bahcesular et al., 2020), that are considered as natural antioxidants. These biomolecules exhibit their activity through various mechanisms, including inhibiting enzymes that inducing free radical produce, increasing endogenous antioxidants, removing free radicals, and inducing the expression of the numerous genes responsible for enzyme synthesis that inhibit oxidative stress (Primiano, Sutter and Kensler, 1997). Ghazwani, Osman and Balamash (2020) have recently reported that the Fourier-transform infrared (FTIR) analysis indicated to increase the content of phenolic acids and flavonoids in basil leaves after treated with 10 kGy of gamma-ray. Moreover, Maraei, Khaled and Elsawy (2017)reported that the gamma irradiation-induced the biosynthesis of certain phenolic compounds.

Also, it seems that gamma irradiation with 10 kGy might stimulate some chemical reactions in basil, which perhaps increase in phenolic content by the breakdown of covalence bonds among phenolic components and, free phenolic components with low molecular weight are increasing (Jamshidi, Barzegar and Sahari, 2014). A complete blood count test is a blood test used to assess general health and detect a range of disorders in hematological parameters. A complete blood count test measures many blood components and features, including red blood cells that carry oxygen, white blood cells that fight infection, hemoglobin that oxygen-carrying protein in red blood cells, hematocrit that indicate to the ratio of red blood cells to the liquid or plasma component of the blood and platelets that help blood clot, and that any change whether an abnormal rise or decrease in the census, indicate the incidence of diseases or disorders requiring medical procedures(Clinic, 2018).

In our study, it has been observed that the levels of HB, RBC, PLT, NEU, and LYM are decreased significantly with a marked increase in the HCT, WBC, MON and reticulocyte count in rats exposed to arsenic compared to the control group. The results are consistent with some studies (Kajiguchi et al., 2005; Bhattacharya and Haldar, 2012; Sumedha and Miltonprabu, 2013; Lemaire et al., 2015; Ghosh et al., 2017; Su et al., 2018).This effect of Arsenic exposure on the hematopoietic system may be attributed to the mechanisms of arsenic toxicity which may induce hemolysis and erythrophagocytosis through increased oxidation of sulfhydryl groups in hemoglobin and decreased oxygen intake by cells as a result of decreased intracellular glutathione, which decreases the lifespan of erythrocytes(Abdul et al., 2015). Moreover, arsenic exposure can also cause a range of changes, such as increasing ceramide formation, membrane disintegration, cytosolic calcium levels, besides decreasing in adenosine triphosphate (ATP) levels, cell membrane integrity affecting erythrocyte lifespan (Abdul et al., 2015).

Regarded the change in platelet count, this confirmed that arsenic inhibited platelet differentiation within the hematopoietic system of bone marrow, leading to reduced platelet production Wu et al., 2014. The white blood cell level was increased in arsenic feed groups be due to the impact of arsenic which induced apoptotic

effect on plasma cells as noted by Rousselot et al. (2004). WBC may generally be divided into five classes, based on their function, morphology and origin: LYM, MON and NEU(Villa et al., 2003). The changes in the LYM and NEU populations present in this study may be due to arsenic caused immune inhibition in rats (Taheri et al., 2016).

Our findings demonstrated that extracts of basil caused improved the disorders that occur in CBC. This is in agreement with the results of previous researches (Ofem et al., 2012; Zangeneh et al., 2019).This effect may due to basil which contains a proportion of iron (Nworgu, Yekini and Oduola, 2013), that contributes to improving the level of HB in the blood and has the ability to stimulate production and increase of RBC to treat deficiency caused by arsenic. Furthermore, in normal, the lack of oxygen in the local tissue appears to lead to the production of glycoprotein known as erythropoietin, which induces increased erythrocyte output(Bowman and Rand, 1980).

Basil leaves extract is very likely to contain erythropoietin-like agents that are responsible for increased erythrocyte production (Ofem, Ani and Eno, 2012). Saha et al. (2012) reported that secondary metabolites of basil, consisting of important elements include essential oil geraniol, a monoterpene and citral, play a role as the modulator in hematological abnormalities (Ofem et al., 2012). Moreover, in results about the increased lymphocyte count after basil administration, it has been reported that *Ocimum basilicum* modulates the cell-mediated as well as a humoral immune response that could be due to the presence of flavonoids and terpenoids (Mediratta et al., 2002).

Antibodies also are known as immunoglobulins, are substances made by the body's immune system in response to foreign substances. Antibodies bind to these foreign substances and they can be killed by the immune system. IgG, IgM and IgA from the major types of antibodies. If reduced levels of antibodies produced by the immune system, it leads to more likely to develop repeated infections(Staff, 2019). The results of our study showed that in the arsenic group of rats (10 mg/kg BW) a highly significant reduction was observed in IgG, IgA and IgM levels compared to the control group. The results are consistent with some studies (Institoris et al., 2001; Sankar et al., 2013). The effect on immunoglobulin levels associated with arsenic exposure can attribute to the arsenic disrupts glucocorticoid regulation, responsible for immune function, (Kaltreider et al., 2001).

Furthermore, the apoptosis caused by arsenic may result in decreased immune responses(Harrison and McCoy, 2001).The administration of water extract from raw or irradiated basil extract demonstrated a protective effect against arsenic toxicity in rats, by increasing antibodies that decreased as a result of arsenic poisoning. These results are in line with the findings of Mohammed, Kadhim and Taher (2017) and Jahejo et al. (2019). Jeba, Vaidyanathan and Rameshkumar (2011)showed that aqueous extract of basil stimulated the antibody production in rats. The flavonoids present in the basil leaves are mainly responsible for the immunomodulatory effect (Ravindran, 2017).

Inflammation is a biological response of the immune system which can be induced by damaged cells, toxic compounds or pathogens (Medzhitov, 2010). It is part of the body's defense mechanism. one of the major aims of inflammation is to bring immune cells to the area of concern as well as to inactivate or destroy any injurious stimuli and to also begin the repair(Ferrero-Miliani et al., 2007; Medzhitov, 2010).The inflammation response is caused by specific immune factors released from the damaged cells. Where, the damaged cells release cytokines, including interleukins, such as IL-6, IL-8, and tumor necrosis factor-α, that are responsible for communication between white blood cells. Interleukins also stimulate the production and release of CRP from the liver; an important component of the innate immune system (Sinclair et al., 2012).

Usually, molecular and cellular activities and interactions efficiently alleviate inevitable infection or damage, during acute inflammatory responses. This effect helps restore homeostasis in the tissue and overcome the acute inflammation. Uncontrolled acute inflammation can become chronic, however, and can lead to a number of chronic inflammatory diseases (Zhou et al., 2016). The elevated levels of inflammatory markers are expected to be associated with toxic metals exposure.Results of this study showed that serum IL-6, TNF-α and CRP level in rats exposed to arsenic was highly significantly elevated. Our findings agree with the results of the previous study on the association between arsenic and ability to cause chronic inflammation by demonstrating the increased pro-inflammatory mediators like TNF-α, IL-6 and CRP in the arsenic exposed group in comparison to the control group(Prasad and Sinha, 2017). Inflammation considered to be one of the main arsenic toxicity mechanisms that can be correlated with increasing cellular damages ,oxidative stress and lipid peroxidation (Bhadauria and Flora, 2007).

In this work also, it was demonstrated that oral treatment with basil extracts diminished inflammations in rats exposed to arsenic. These results in agreement with Aye et al. (2019) and Takeuchi et al. (2020) who found that basil has anti-inflammatory effects. Rodrigues et al. (2016) reported that the basil essential oil was effective in reducing inflammations (acute or chronic) by induced inhibiting of the inflammatory mediator receptors and the migration of cells to stimulus locations. This may be attributed to the contains of basil of rosmarinic acid (Kwon et al., 2019), where this compound has been related to anti-inflammatory activities (Luo et al., 2020).

Antioxidants are considered the enzymes of the body's protection, are able to stabilize free radicals before attacking components of the cell. They work to diminishing free radicals through reducing their energy or donate electrons to them, thus making it stable(Krishnamurthy and Wadhwani, 2012). While,

"oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses"(Betteridge, 2000). In the present study, observed a very highly significant decrease in GSH, SOD and CAT accompanied by increased MDA of the rat's brain in the arsenic group as compared to the normal control group. This result is similar to the study of Sun et al. (2018)that reported that arsenic caused significantly decreased GSH, SOD and CAT with increased MDA content in the brain tissues of chickens. This may be attributed to the toxic effect of arsenic that may induce oxidative stress by interacting with antioxidants, resulting in the accumulation of free radicals in cells (Bonetto, Villaamil Lepori and Puntarulo, 2014).

In contrast, basil giving a positive effect on the brain by improving the levels of antioxidant antioxidants, and fat oxidation (MDA). These results were in line with recent studies showed that the water extract of gamma-irradiated basil contributes to improving the oxidative stress induced by arsenic exposure in rats (Ghazwani, Osman and Balamash, 2020; Osman, Ghazwani and Balamash, 2020). Also agree with the results of Khodabakhshi et al. (2017), who proved that the increased level of MDA in the mice brain tissue following seizures was prevented by basil extract. Moreover, Khaki (2016) demonstrated that the basil extract protects brain cells from the harmful effects by regulating the antioxidant enzymes in the serum. This saves the neurons from irreversible cell injury. The antioxidant effect is due primarily to phenolic elements, such as, phenolic acids and flavonoids, which have redox properties and ability to neutralize free radicals (Shahidi et al., 1992).

CONCLUSION

In this study, we demonstrated that gamma radiations have the ability to increases antioxidants contents in basil leaves. Moreover, the water extracts obtained from *Ocimum basilicum* can be successful in diminished arsenic toxic effect through the improvement of the Blood homeostasis of CBC and immunoglobins level, reduced inflammatory markers and, enhancement ability of antioxidants to be overcome oxidative stress in the brain.

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Neuroprotective Role of Diosmin on Rotenone Induced Neurotoxicity in SH-SY5Y Neuroblastoma Cells

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ABSTRACT

Diosmin, a naturally occurring citrus flavonoid glycoside, has been reported to offer neuroprotection by its antioxidant, anti-inflammatory, neuroprotective, anti-cancer, and antiapoptotic properties. The present study aims to elucidate the underlying neuroprotective mechanism of Diosmin in rotenone-induced neurotoxicity. The SH-SY5Y cells were used to study the cell viability assay and determined by using MTT assay with different concentrations of rotenone (5, 10, 50,100, and 200 nM) and Diosmin (5 nM, 10 nM, 20 nM, 50 nM,100 nM, 200 nM, 500 nM, 1 µM, 10 µM, 100 µM, 200 µM, and 500 µM) for 24 h and MTT assay was performed to detect IC50 value of rotenone and Diosmin. The Cell viability was assessed by exposing SHSY5Y cells to various concentrations of rotenone (5–200 nM) for 24 h. The therapeutic effect of Diosmin (5–200 nM) against rotenone was measured by pre-treatment of Diosmin at various concentrations and then incubation with rotenone (100 nM). Using an effective dose of diosmin (100 nM), mitochondrial membrane potential, levels of reactive oxygen species (ROS), and expression patterns of apoptotic markers were assessed. The toxicity of rotenone was accompanied by the loss of mitochondrial membrane potential, increased ROS generation, the release of Cyt-c, and enhanced expressions of pro-apoptotic and downregulation of anti-apoptotic. Our results indicated that the pre-treatment of Diosmin attenuated rotenone-induced mitochondrial dysfunction, oxidative stress, and apoptosis. Thus, Diosmin may serve as a potent therapeutic agent in the future by its multiple pharmacological properties in the treatment of neurodegenerative diseases including PD.

KEY WORDS: DIOSMIN, NEUROPROTECTIVE, PARKINSON'S DISEASE.

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INTRODUCTION

Parkinson disease (PD) is the most common neurodegenerative and movement disorder, that is characterized by resting tremor, rigidity, postural abnormalities, stooped posture, bradykinesia, akinesia, and festinating gait. These motor deficits have been mainly attributed to the progressive loss of neuromelanin containing dopaminergic neurons in substantia nigra (SN)

and lead to the loss of dopamine (DA) in the striatum and manifest as motor disabilities that are characteristics of PD (De Lau and Breteler, 2006). The etiology of PD remains elusive and comprises the involvement of both genetic and environmental factors. Agricultural pesticide use has been well-established causes of parkinsonism (Tanner and Goldman, 1996). One among them is the neurotoxin rotenone; a lipophilic molecule that easily crosses the cellular membranes and blood-brain barrier.

Exposure of rotenone mimics numerous pathological features of PD, including dopaminergic neuronal death, mitochondrial dysfunction, oxidative stress, inflammation, and behavioral changes that possess *in vitro* and *in vivo* studies (Hoehn and Yahr, 1967; Al-Adawi et al. 2000; Shulman et al. 2001). By inhibiting mitochondrial complex, I activity, rotenone generates reactive oxygen species (ROS), inhibits adenosine triphosphate (ATP) synthesis, depolarizes the mitochondrial membrane, and finally leads to the death of neurons in SN (Pollanen et al. 1993). ROS produced from mitochondrial dysfunction causes changes in the mitochondrial membrane permeability, leading to the activation of apoptotic caspases (Kuzuhara et al. 1988). Plants and based bioactive compounds play an important role in the production of medicine for various diseases and disorders (Rajeshkumar et al. 2019, Renukadevi Balusamy et al. 2020, Thangavelu et al. 2020, Mehta et al. 2020, Tamizhselvi et al. 2020, Rajakumari et al. 2020).

Currently available pharmacological interventions provide symptomatic relief for patients with PD and have little efficacy in reversing the underlying neuropathological changes associated with the disease. Therefore, there is a clinical need to identify therapeutic agents that can ameliorate, or slow down the deleterious processes associated with PD. One such paradigm is to explore the possible contribution of natural products that might interfere with PD pathology (Al-Maskari et al.2011). Natural products have been increasingly found to have specific molecular or pharmacological effects that are likely to contribute to the development of neuroprotective agents against PD. Diosmin, (3′, 5, 7-Trihydroxy-4′-methoxy flavone 7-rutinoside) is found naturally in numerous plants, including citrus fruits, especially lemons, green Meyer lemons, and Buddha's finger fruits *Caucasian vetch, Hyssopus officinalis*, and *Hyssopus*.

Also, it is found quite effective in mitigating hyperglycemia in diabetic rats (Pari and Srinivasan, 2010). It is also speculated that diosmin has neuroprotective potential in the treatment of Alzheimer's disease (Mustafa, et al. 2010; Essa et al. 2012), and against lipopolysaccharide-induced neurotoxicity in PC 12 cells (Halliwell and Whiteman, 2004). However, the neuroprotective effect of diosmin against experimental PD is not investigated so far. Because of this light, the present study was aimed to evaluate the neuroprotective effect of diosmin against rotenone-induced *in vitro* model of PD.

MATERIAL AND METHODS

Chemicals: Rotenone, Diosmin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-7-diacetyl dichlorofluorescein (DCFH-DA), rhodamine 123 (Rh-123), heat-inactivated fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), antibiotic/antimycotic, EDTA, and Trypsin-EDTA were procured from Sigma Chemicals Co. (St. Louis, USA). Anti-Bcl-2, anti-Bax, caspase-3, caspase-8, caspase-9, Cyt-c, and anti-JNK and anti-P38 MAPK antibodies were obtained from Cell Signaling (USA) and β-actin, anti-mouse, and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA).

Cell Culture: SH-SY5Y cells were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were grown in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution. Cultures were maintained in a humidified incubator at 37 in an atmosphere of 5% CO2 and 95% air. The cell culture medium was changed every 2 days.

Cell Viability Assay: Cell viability assay was determined by MTT assay, as described previously (Johnson et al. 1980). SH-SY5Y cells were collected and seeded in 96-well plates, at a density of 3 ×103 cells/well. To determine the toxicity of rotenone, cells were incubated with different concentrations of rotenone (5, 10, 50,100, and 200 nM) and Diosmin (5 nM, 10 nM, 20 nM, 50 nM,100 nM, 200 nM, 500 nM, 1 µM, 10 µM, 100 µM, 200 µM, and 500 µM) for 24 h and MTT assay was performed to detect IC50 value of rotenone and Diosmin. To assess the therapeutic efficacy of Diosmin against rotenone toxicity, cells were pre-treated with different concentrations of Diosmin (5,10, 20, 50, 100, and 200nM) for 2h and then incubated with rotenone (effective dose) for 24 h. Diosmin was also present during rotenone treatment for an additional 24 h. Then all the cells were incubated with MTT final concentration (1mg/mL of serum-free DMEM medium) at 37•C for 4h. After the incubation, the medium was removed, and 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance of the formazan product was evaluated by a spectrophotometer at 570 nm using a microplate reader. Four independent experiments were performed from each group.

Based on the results obtained from cell viability assay, the effective dose of Diosmin against rotenone toxicity was utilized to study the effect of Diosmin by assessing ROS, MMP, apoptosis, and apoptotic markers protein expression.

Experimental Design (n= 4 Experiments): Group I: untreated control cells, Group II: rotenone (effective dose: 100 nM), Group III: Diosmin (100 nM) + rotenone (100 nM), Group IV: Diosmin (100 nM).

Measurement of Intracellular ROS: The levels of endogenous ROS formed in control and experimental cells were estimated by using fluorescence dye (DCFH-DA) (Jayaraj et al. 2013). After pre-treatment with Diosmin (100 nM/mL) for 2 h, the cells (1 ×105 cells/well in 6-well plates) were incubated with rotenone(100nM/mL) for 24 h and then incubated with 100 µL DCFHDA for 30 min at 37•C and washed twice with PBS to remove the excess probe; the cells were suspended in glucose enriched PBS and transferred and visualized using a fluorescent microscope. Fluorescent measurements were made with excitation and emission filters set at 485 ±10 nm and 530 ± 12.5 nm, respectively (Shimadzu RF-5301PC spectrofluorimeter), and the images were captured using a fluorescence microscope (Hu et al. 2009).

Measurement of Mitochondrial Transmembrane Potential (MMP): MMP changes were determined by the mitochondrial-specific, incorporation of a cationic fluorescent dye Rh-123 (Bernheimer et al. 1973). After treatment with Diosmin for 2 h and rotenone for 24 has previously been described, the cells (1 ×105 cells/well in 6-well plates) were changed to fresh medium containing 1 µL of fluorescent dye Rh-123 (5mmol/L) and kept for 30min at 37•C. The cells were then collected, washed twice with PBS, and estimated by using a blue filter (450–490 nm) (Shimadzu RF-5301 PC spectrofluorimeter).

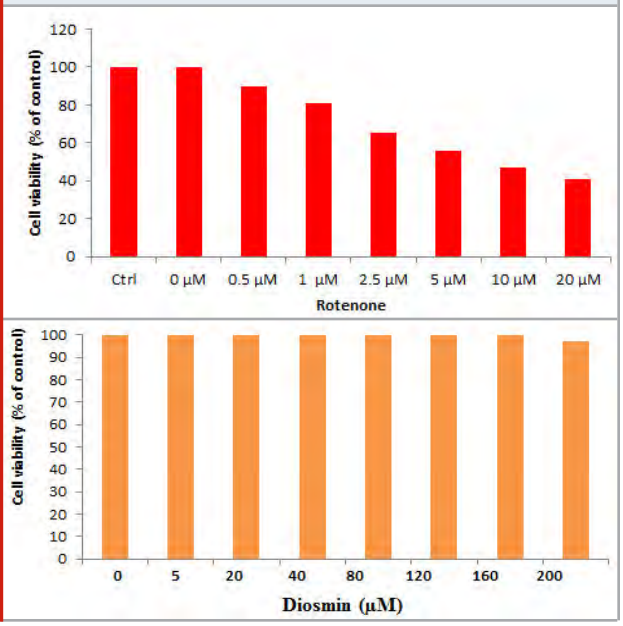
Apoptosis Analysis Using Dual Staining: Dual staining method is used to analyze the apoptotic morphological changes by treating the control and experimental cells with fluorescent probes acridine orange and ethidium bromide (AO and EB) and using a fluorescence microscope (Lirdprapamongkol et al. 2005). After the treatment schedule as described in previous experiments, the medium was removed from the plates; cells (1 × 105) were washed with PBS twice and then fixed with 4% paraformaldehyde for 20 min and stained with 100 µg/mL AO and EB. These cells were incubated for 20min at room temperature and washed with warm PBS to remove excess dye. Cellular morphology was examined using fluorescence microscopy and photographed and quantified.

Data Analysis: Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean ± SD for four experiments in each group. <0.05 were considered significant.

RESULTS AND DISCUSSION

Cytotoxicity of Rotenone in SH-SY5Y Cells: A dose-dependent cytotoxic effect of rotenone was evaluated by MTT assay in SH-SY5Y human neuroblastoma cells, which measures mitochondrial function or integrity with a dose of 100 nM which caused ~50% of cell death as compared with controls and was taken as inhibitory dose (Figures 1(a) and1(b)).

Figure 1: Effect of Diosmin on rotenone-induced cytotoxicity in SH-SY5Y neuroblastoma cells were assessed by MTT assay. (a) shows the dose-dependent effect of rotenone (5, 10, 50, 100, and 200 nM) induced cell toxicity after 24 h. An approximately half-maximal inhibition of cell viability was obtained at 100 nM rotenone concentration. (b) shows the dose-dependent effect of Diosmin at various concentrations. Low concentrations (5, 10, 20, 50, 100, and 200 nM) did not induce any toxicity after 24 h treatment, whereas slight toxicity was induced at 500 µM concentration. Values are expressed as the percentage of the untreated control and represented as mean ± SD of four independent experiments in each group.



3.2. Diosmin Protects Rotenone Induced SH-SY5Y Cell Death: Figure 2 shows the protective effect of diosmin against rotenone-induced injury (100 nM) with cell viability increasing to 84 ± 6.7% of control in the presence of 100 nM Diosmin. So, based on the dose-response data, the treatments of 100 nM Diosmin and 100 nM rotenone were chosen for further experiments (Figure 2).

Diosmin Attenuates Rotenone Induced ROS Generation: To analyze the effect of Diosmin on free radical generation, the levels of intracellular ROS formed were

quantified by fluorescence with H2DCF-DA. Rotenone treatment enhanced the green fluorescence, an indicator of high levels of ROS, and pre-treatment of Diosmin to rotenone exposed cells revealed reduced green color intensity, an indicator of decreased ROS formation (Figures 3).

Figure 2: The protective effect of Diosmin (5, 10, 20, 50, and 100 nM) against rotenone-induced cell death was determined by the MTT assay. Values are expressed as the percentage of the untreated control and represented as mean ± SD of four independent experiments in each group.

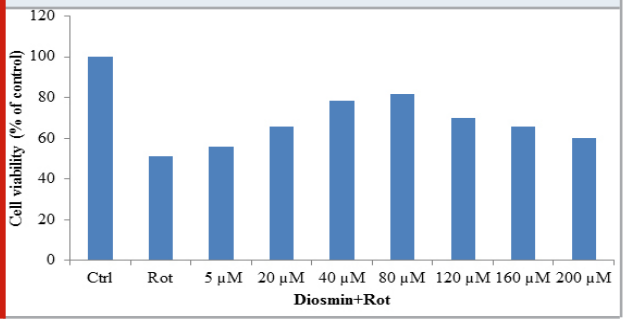
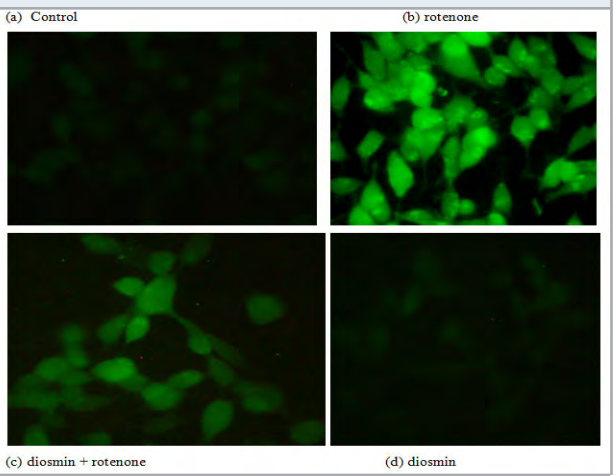
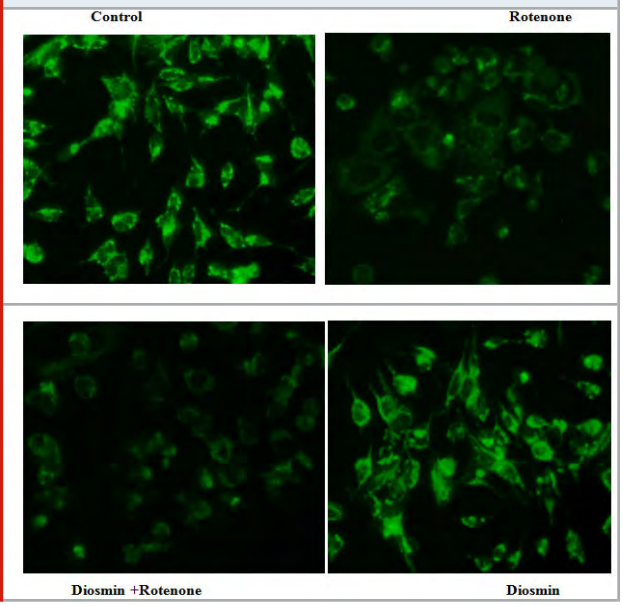


Figure 3: Diosmin reduced ROS formation as stained by 1 µM CM-H2 DCFDA. (a) Photo-micrograph showing the preventive effect of diosmin (100 nM) against rotenone induced ROS generation. (A)Control, (B) rotenone, (C) diosmin + rotenone, and (D) diosmin.



Diosmin Ameliorates Rotenone Induced Mitochondria membrane potential (ΔΨm): Alteration in the MMP is considered to be one of the important events related to apoptosis. The effect of Diosmin on MMP in rotenone-induced toxicity was by measuring the uptake of Rh-123. In normal cells, Rh-123 steadily penetrates the cells, stains mitochondria, and exhibits high fluorescent intensity. The depolarization of MMP due to rotenone treatment results in the loss of Rh-123from the mitochondria and a decrease in intracellular green fluorescence. Cell cultures pre-treated with diosmin before rotenone treatment partially reduced this decline in fluorescence and approached control levels (Figure 4).

Figure 4: Diosmin stabilizes MMP as stained by Rh-123. Rotenone (100 nM) significantly decreased mitochondria membrane potential, while cells that were pre-treated with diosmin (100 nM) significantly increased MMP. Values are given as mean ± SD of four independent experiments in each group. p< 0.05 compared to control; #p <0.05 compared to rotenone groups (DMRT).

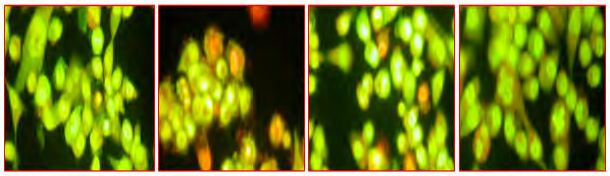


Effect of Diosmin on rotenone-induced apoptotic features in SK-N-SH cells by dual staining: Apoptotic morphological changes were measured in terms of fluorescence by Acridine orange (AO) and Ethidium bromide (EtBr). Our results indicated that the Control cells which fluoresced brightly with green nuclei and normal morphology were showed in Figure 6(a) and the rotenone (100 nM) treated cells exhibited significant nuclear fragmentation and destruction which is characteristic of Apoptosis (bright orange color) and necrosis (red color), respectively. However, the amount of fragmentation and destruction of rotenone treated cells were dramatically reduced when the cells were pre-treated with Diosmin (5 µM) (Fig. 5a &tb).

Figure 5 clearly shows the diosmin protects SH-SY5Y cells against rotenone-induced apoptosis. (a) Photomicrograph showing the antiapoptotic effect of (100 nM) against rotenone at a concentration of 100nM effective dose. (A) Control, (B) rotenone, (C) diosmin + rotenone, and (D) diosmin. (b) Rotenone (100 nM) treatment induced cell apoptosis compared to control cells; pre-treatment with diosmin (100 nM) suppresses these apoptotic features. Values are given as mean ± SD of four independent experiments in each group. P< 0.05 compared to control and #P< 0.05 compared to rotenone group (DMRT). Results of the present study indicated

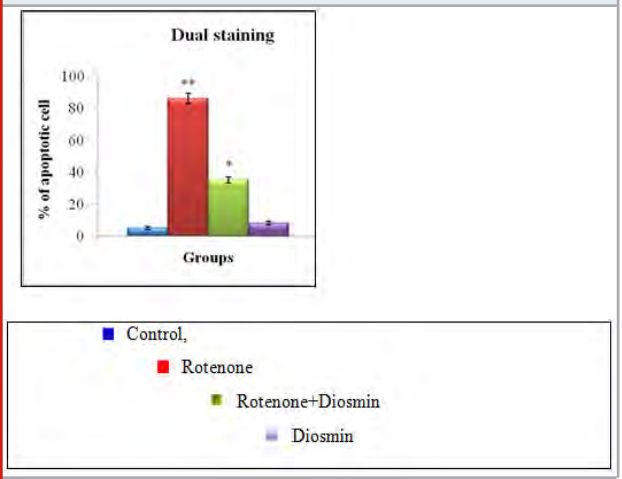
that the rotenone treatment for 24 h destroyed SH-SY5Y cells in a dose-dependent manner and approximately half-maximal inhibition of cell viability (54.41%) was obtained at 100 nM rotenone concentration, which corroborated our previous studies (Kim H. J et al. 2007). The observed condition mimics the situation at the time of initial diagnosis of PD, when approximately 50% of neurons in the substantia nigra are alive, although many of them may be undergoing subcellular stress (Bender et al. 2006).

Figure 5: (A) Photo micrograph illustrates apoptotic morphological changes in SK-N-SH cells treated with diosmin and rotenone.



(a) Control (b) Rotenone (100 nM) (c) Diosmin + Rotenone (d)Diosmin (5 µM)

Figure 5(B): depicts apoptotic morphological changes in control, TF and rotenone treated SH-SY5Y cells. Values are given as mean±SD of four experiments in each group. **P<0.05 compared to non-treated cells; *P < 0.05 compared to rotenone-treated cells.



In the present study, 24 hours prior exposure of diosmin significantly enhanced cell viability in a dose-dependent manner. In this study, the IC50 of diosmin was found at 100 nM. In the present study, increased levels of ROS observed in the rotenone model indicated that oxidative stress was induced by rotenone and is attenuated by treatment of diosmin which might be because of its free radical scavenging activity. Though diosmin is a potent antioxidant, its alone treatment to SH-SY5Y cells triggers the levels of ROS non significantly. Results of the present study corroborate previous experiments, in which the addition of celastrol, a triterpenoid (Seaton et al. 1997), rutin, a quercetin glycoside (Talpade et al. 2000), and baicalein, a flavonoid (Koopman et al. 2010), alone increased the levels of ROS. Treatment with diosmin ameliorated 3-nitropropionic acid-induced impaired mitochondrial enzyme complexes (I, II, and IV) in an experimental model of Huntington's disease (Turrens, 2003). Further, it could inhibit singlet oxygen-induced protein and lipid oxidation (Turrens. 1997; Kamat et al. 2000; Santosh Kumar et al. 2002; Song et al. 2012; Choi et al. 2014; Park et al. 2014).

CONCLUSION

In conclusion, this study demonstrates that Diosmin, a black tea polyphenol exhibited neuroprotective effects on rotenone-induced apoptosis in SH-SY5Y cells. These antiapoptotic effects of Diosmin appeared to be related to its ability to reduce oxidative stress and maintain the MMP stability in SH-SY5Y cells against rotenone toxicity. Hence, Diosmin may be a potential agent for the treatment of neurodegenerative disorders such as PD, though further research in *in vitro* and experimental models (in vivo) are needed to prove its neuroprotective effects.

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Conflict of Interests: Herewith all the authors declare that they do not have any conflict of interests.

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Methods of Deodorizing Round Scad (*Decapterus maruadsi*) for the Production of Protein Hydrolysates

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ABSTRACT

Round scad, a type of sea food naturally has a disadvantage of the fishy smell, which unfavorably affects the sensory quality. In this study, the sensory parameter, trimethylamine (TMA) indicator, and amino acid values were evaluated in various methods during fishy-smell removal from round scads (*Decapterus maruadsi*) as the material for bouillon cube production. Salt, lactic acid, and ethanol in different concentrations were used as deodorizing agents for the material round scads. Round scad hydrolysate was prepared by using tea (1%), ethanol (1%), and activated charcoal (1.5%). Besides, *Lactobacillus plantarum, Saccharomyces cerevisiae* were also used for the preparation. The different deodorizing methods provided distinctive results. Deodorization was most effective when raw materials were pretreated with 0.03% acetic acid and 0.1% salt and followed by fermentation of scad hydrolysates with *L. plantarum* (1%, v/v) for 12 hours. The treated hydrolysate had a sweet taste, light brownish yellow color, and no fishy odor remaining. The lowest TMA obtained analysis was 3.05 ± 0.38 mg/100g, and the highest amino acid was 13.76 ± 0.3 g/l.

KEY WORDS: *DECAPTERUS MARUADSI*, ROUND SCAD, FISHY ODOR, TRIMETHYLAMINE, HYDROLYZE.

INTRODUCTION

Round scads (*Decapterus maruadsi*) is a species of oceanic fish in the family Carangidae. Round scads are economically valuable and a common harvested resource in the world. Round scads have high nutritional value, containing an average protein value of 18-20%, lipid 0.5-0.8%, ashes 1.3^{-1}.5%, mineral 58-246 mg/100g (including Ca, P, Fe, K...), and various kinds of vitamins such as A, B1, B2, and C (Nguyen, 2011).Round scads, like other kinds of seafood, had a fishy odor, generally caused by NH_3, TMA, TMAO, indol, and skatol (Deke et al., 2016). The smell severely affects the sensory characteristics, thereby reducing the market value and the commercializing of value-added products made of round scad, such as hydrolysate powders.

There were various studies worldwide to deodorize the fishy smells by physical, chemical, or biological factors, such as activated charcoal, polyphenol in tea, acetic acid, ethanol or yeasts and bacteria (*Saccharomyces cerevisiae*, *Lactobacillus plantarum, L. bulgaricus,* and *Streptococcus thermophilus*). The absorbent of activated charcoals helps effectively eliminate the volatile aldehydes and ketone. Tea reduces the relative aldehyde values significantly while increases the akan, as well as adequately inhibit the

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formation of trimethylamine (TMA) in the hydrolysates (Deke et al., 2016). Ethanol, methyl, and ethyl alcohol, or ethyl acetate are among the productive deodorizers for fish and oysters (Cuellar et al., 2017; Galliver and Holmes, 1957; Levin, 1961). Some authors reported the effect of acetic acid in deodorizing such as Bui (2013), Nguyen (2000a), Nguyen (2000b), Nguyen and Nguyen (2017). Deke et al. (2016), in a study, pointed out that the yeast S. cerevisiae deodorized by increasing the relative contents of aromatic compounds and decreasing the relative contents of aldehydes and ketone. Fermentation with the bacteria *L. plantarum* was also reported to be effective in improving the smell and taste of fish and oysters' hydrolysates (Lee et al., 2016; Pan et al., 2018).

However, there has not yet been a study to deodorize round scad to produce hydrolysate powder. Without the odor treatment, the smelling factors remaining in the raw material would reduce the sensory characteristics of the protein hydrolysate powder product. Deodorizing is an essential step to overcome the disadvantage of the material and optimize the quality of the final product. Therefore, we conducted this study to investigate the deodorization of round scads for producing fish protein hydrolysates.

MATERIAL AND METHODS

Materials for the research: Round scad: The raw round scad materials (*Decapterus maruadsi*) were purchased in May-Chai fish port, Hai Phong province. The materials had the particular fishy smell, were fresh, sheen, intact, in the size of 12-14 fish per kg, and 14-15cm long per fish. The materials were cleaned, packed in 0.8-1.0 kg plastic packages, and stored at -20 ± 2°C in the lab. Ingredients and spices used in the study, such as salt, acetic acid, ethanol, were conformed to the standards of ingredients for food production No. 02/VBHN-BYT from the Ministry of Health.

Figure 1: Round scads raw materials

Experimental design: The experiments were designed to change one factor and keep the other factors unchanged. The result of one experiment would be used as a constant in the next experiment. The experiments were repeated 3 times.

Experiments for materials deodorizing before hydrolyzing: Viscera and gills were removed from the material round scads before used. The round scads were then washed with different solutions: (1) salt solutions from 0-1% (in 0.25% increment); (2) acetic acid solutions from 0-0.05% (in 0.01 increment); (3) ethanol solutions from 0-3% (in 0.5% increment). The solution temperature was controlled at 10 ± 2°C. The washing time was 15 minutes. The deodorizing effect was evaluated using sensory assessment (smell), nitrogen amino acid, and TMA value.

Experiments for deodorizing during hydrolysis: Preprocessed round scads were minced and hydrolyzed using protease. The deodorization was tested with five different factors including dried tea (1% w/w), ethanol (1% w/w), activated charcoal (1.5% w/w), the bacteria *Lactobacillus plantarum* (1% v/w), and the yeast *Saccharomyces cerevisiae* (1.5% v/w). The effects of the deodorization were evaluated by sensory assessment (smell, color, state), amino acid nitrogen (Naa), and TMA values of the hydrolysates.

Methods of analysis: TMA was determined using the descriptive analysis method, according to Woyewodal et al. (1986). Total nitrogen (Nts) was done according to TCVN 3705-1990; amino acid nitrogen (Naa) according to TCVN 3708-1990. The sensory assessment was done using a scoring system from a five member committee, according to TCVN 3215-79, and the sensory tests were according to TCVN5277-1990.

Table 1. Material round scad smell scoring scale

No.	Smell characteristics	Score	Rank
1	No more fishy smell, no treating solution smell	5	Good
2	Little fishy smell, no treating solution smell	4	Fair
3	Little fishy smell, tint of treating solution smell	3	Medium
4	High fishy smell, no treating solution smell	2	Bad
5	Very high fishy smell, spoiled or having treating solution smell	1	Very bad

Data analysis: Each experiment was repeated 3 times. Data processing and chart drawing were done using Microsoft Office Excel 2007.Data analysis was done using IBM SPSS Statistics 20.

RESULTS AND DISCUSSION

The sensory assessment results after preprocessing the round scad raw materials with different salt concentration solutions is presented in Table 3.

The results showed that salt preprocessing provided better sensory assessment scores while not causing noticeable changes in Naa. The best sensory quality was obtained in

the formulas from 1% salt and above. However, apparent fishy smells were remaining in all concentrations, which were suggested by the results of the TMA value. TMA results were not significantly reduced compared to the control sample (10.56 mg/100g as opposed to the lowest value among formulas, 10.077mg/100g). There was no statistically significant difference among salt concentrations ($p>0.05$) (Figure 2).Therefore, 1% of salt concentration to be the suitable for preprocessing round scad raw material.

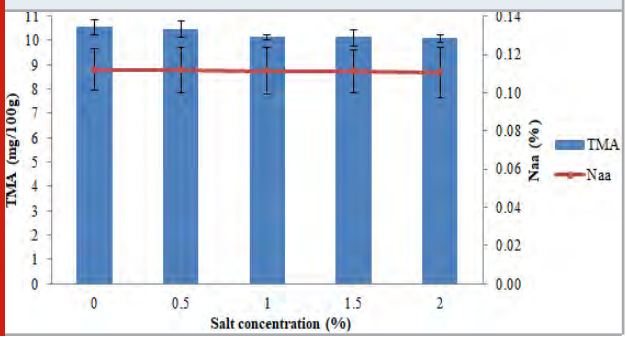
Table 2. Round scad hydrolsates sensory scale

Parameter	Score					Weigh Score
	5	4	3	2	1	
Color	Light golden brown	Light yellow	Brown	Dark brown	Greyish black	0.6
Smell	Specific scad smell, no fishy odor, no strange odor	Light fishy smell, no treating solution smell, no strange odor	Light fishy smell, mixed with treating solution smell or strange odor	Clear fishy smell, with or without treating solution smell or strange odor	Strong fishy smell, spoiled or ill odor	1.6
Taste	Sweet, having after taste, no strange taste	Sweet, having after taste, mixed with strange taste	Light sweet, with or without strange taste	Not sweet, not bitter, with or without strange taste	Bitter, not sweet, mixed with strange taste	1.2
State	Clear solution, diluted, not viscous	Clear solution, diluted, viscous	Slightly cloudy, little precipitated, diluted.	Slightly cloudy, little precipitated, viscous	Opaque, a lot of preciprited, very viscous	0.6

Table 3. The sensory assessment results with different salt concentration

Formula	Salt concentration (%)	Average sensory scores ± SD
1	0	2.333 ± 0.577
2	0.5	2.667 ± 1.155
3	1.0	3.000 ± 1.000
4	1.5	3.000 ± 0.000
5	2.0	3.000 ± 1.000

Figure 2: TMA and total amino acid (Naa) values against different salt concentration (in average value ± SD)

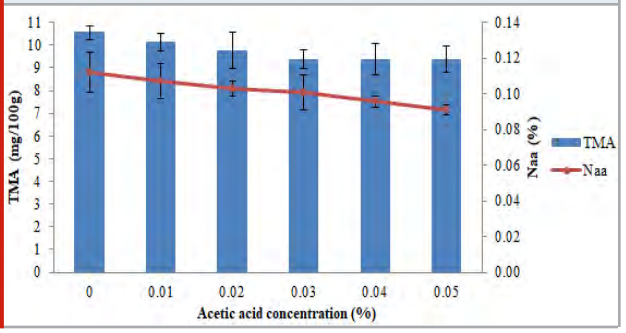


Determining the concentration of acetic acid in preprocessing round scad materials: The sensory assessment results of different acetic acid concentrations are presented in Table 4 and the corresponding TMA and Naa values in Figure 3.

Table 4. The sensory assessment score (average value ± SD) for different acetic acid concentration treatments

Formula	Acetic acid concentration (%)	Sensory scores
1	0	2.333 ± 0.58[a]
2	0.01	2.667 ± 1.16[a]
3	0.02	3.000 ± 1.00[a]
4	0.03	3.667 ± 0.58[a]
5	0.04	3.333 ± 0.58[a]
6	0.05	2.667 ± 0.58[a]

Figure 3: TMA and total amino acid (Naa) values against different acetic acid concentration (in average value ± SD)



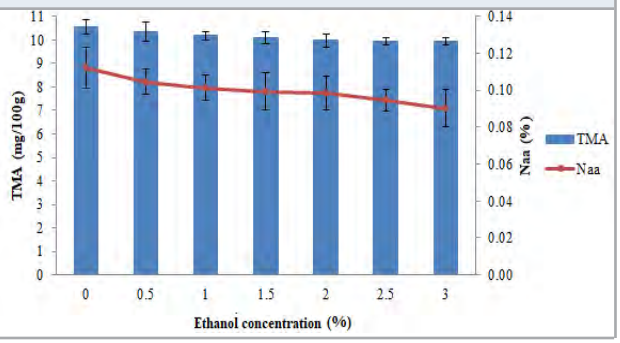
When the acetic acid concentration was increased from 0.01% to 0.05%, the fishy odor and Naa. were decreased. The acetic acid concentration of 0.03% provided the

most decrease in the material's smell, the highest sensory score, and slight Na.a reduction compared to the control sample ($p>0.05$). On the other hand, when acetic acid concentration continued to rise to 0.05%, there was no significant change in TMA values as opposed to that of level 0.03%. At the same time, Naa was lost, and sensory scores were considerably reduced due to the acetic acid smell in materials (Figure 3). Therefore, a suitable acetic acid concentration for round scad material processing to be 0.03%. Although using acetic acid could reduce the fishy smell, there were still slimes on the surface of the materials. Therefore, it was necessary to combine with salt to gain maximum effectiveness.

Table 5. The sensory assessment score (average value± SD) using different ethanol concentrations

Formula	Ethanol concentration (%)	Sensory score
1	0	2.333 ± 0.577[a]
2	0.5	2.667 ± 0.577[a]
3	1.0	3.000 ± 1.000[a]
4	1.5	3.000 ± 1.000[a]
5	2.0	3.333 ± 0.577[a]
6	2.5	3.000 ± 0.000[a]
7	3.0	3.000 ± 1.000[a]

Figure 4: TMA and total amino acid (Naa) values against different ethanol concentration (in average value ± SD)



Determining the concentration of ethanol in preprocessing round scad materials: The sensory assessment results of different ethanol concentrations are shown in Table 5 and the corresponding TMA and Naa values in Figure 4.

The different ethanol concentrations provided mixed results. Although there was no divergence in the sensory assessment among formulas, when the level of ethanol increased, the TMA and Naa values fell. The highest effective concentration was 2% ethanol, by which the TMA value was 9.983 mg/100g, and Naa had no significant reduction compared to the control ($p>0.05$). However, all formulas showed ineffective results as the smell remained clear (Figure 4).In all three experimented methods, round scad preprocessing with acetic acid 0.03% provided the highest results. Therefore, using a combination of acetic acid 0.03% and salt 1% for preprocessing the scad material.

Deodorizing during round scad hydrolysis: The sensory scores from the various methods provided divergent results, as presented in Table 6 and the corresponding TMA and Naa values in Figure 5.

All methods showed some deodorizing abilities to the hydrolysate with statistically different TMA values ($p<0.05$). The TMA values in the activated charcoal, ethanol, and yeast methods were reduced 25-30% from the control sample, in the range of 8-9mg/100g. The tea treatment method provided a strong deodorization with a TMA value of 5.01 mg/100g. The fermentation with Lactobacillus plantarum in 12 hours resulted in the highest effectiveness, which totally excluded the fishy smell and gave the lowest TMA value of 3.05 mg/100g, four-fold reduced from the control. The method also brought the obtained amino acid value to 13.76g/l compared to 12.55g/l from the control sample. In contrast, other methods showed no statistically meaningful effect on amino acid value ($p<0.05$), except for activated charcoal and ethanol, which lowered amino acid levels in the hydrolysate to 11.29 and 12.09 g/l, respectively (Figure 5).

In the hydrolysate production process, material preprocessing plays an essential role as it not only cleans any unfavorable substances potentially reduces the product quality but also eliminates odor and brightens the resulting hydrolysates. The basis of the deodorization is the reactions that change the nitrogen in the smell causing substances, therefore, decrease or eliminate their odors (Doyle and Glass, 2010).Adding salt into the cleaning solution helps eliminate slimes, smelling substances, and neutralize impurities. The Cl- ions in salt solution attach to and inactivate the proteases on the surface of materials, thus inhibiting the spoilages that cause odors (Doyle and Glass, 2010).The smelling factors in seafood typically have weak alkalic nature such as NH3, TMA, TMAO, indol, skatol; therefore, weak organic acids can be used to neutralize these substances and change them to salt compounds, so that can reduce or exclude the smell.

Acid solutions also eliminate bacteria that cause smelling. Among organic acids, acetic acid is an economical and accessible de-colorant and deodorant, effective even in a small concentration, and does not create any unfavorable effect on human health (Bui et al., 2003; Nguyen and Nguyen, 2017). In acidic treatment, TMA was neutralized as following:

$$(CH_3)_3N + RCOOH \rightarrow (CH_3)_3NH + RCOO-$$

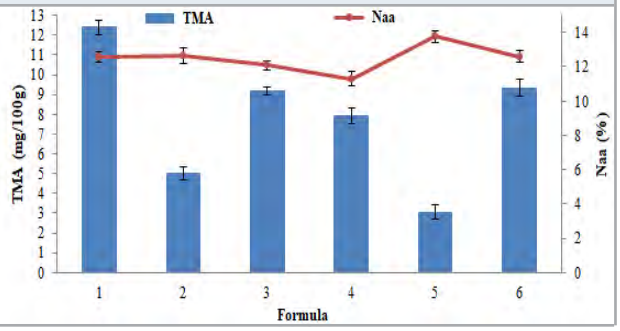
Bui et al. (2013) deodorized Acetes using acetic acid 0.03%. Nguyen and Nguyen (2017) in their study to utilizing the heads and bones of Yellowfin tuna fish to produce tuna floss, used acetic acid 0.03% to treat the smell. There are other studies which have not yet been published such as deodorizing shark meat using acetic acid 0.2% (Nguyen et al. 2000a), deodorizing cyprinid fish in surimi production using salt solution and acetic acid 0.05-0.15% (Nguyen, 2000b). In our study, we

decided that combining salt solution (1%) and acetic acid (0.03%) provided the optimal effect in deodorizing and eliminating the slime on the surface of the materials. Galliver and Holmes (1957) suggested to effectively deodorize fish meat using alcohol. Levin (1961) also proposed the method of fish products deodorizing using methyl and methyl alcohol, or ethyl acetate. To eliminate the fishy smell of algae, Cuellar et al. (2017) used ethanol. However, in our study, ethanol was not as effective as acetic acid, and was not chosen.

Table 6. Deodorizing results during hydrolysis (in average value ± SD)

Formula	Method	Sensory assessment	Score
1	Control	Dilute solution, clear, golden brown color, light sweet, clear fishy odor	10.267 ± 0.306[a]
2	Dried Tea	Dilute solution, clear, light brown color, sweet with an aftertaste, light acrid, slightly fishy, having a pleasant aroma	15.600 ± 0.200[b]
3	Ethanol	Dilute solution, clear, golden brown color, sweet, clear fishy smell	11.867 ± 0.306[c]
4	Activated charcoal	Dilute solution, clear, light yellow color, slightly sweet, slightly fishy smell	14.667 ± 0.231[d]
5	*Lactobacillus plantarum*	Dilute solution, clear, golden brown color, sweet with an aftertaste, no fishy odor, having a light pleasant aroma.	18.267 ± 0.416[e]
6	*Saccharomyces cerevisiae*	Dilute solution, clear, golden brown color, slightly sweet, clear fishy odor mixed with an unpleasant smell	11.333 ± 0.416[c]

Figure 5: TMA and total amino acid (Naa) values against different deodorization methods (in average value ± SD)



Deke et al. (2016) studied the effect of activated charcoal, yeast extract, and polyphenol in tea on *Paphia undulata* hydrolysate. The results showed that tea was the most effective, with TMA as low as 3mg/100g. The highest TMA was when using yeast extract (around 9 mg/100g). Feng (2009) used activated charcoal to deodorize oyster hydrolysates. In his study of sardine oil deodorization, Chakraborty et al. (2014) used activated charcoal 1.25-3.75% in 40 minutes with high efficiency. The activated charcoal with a hollow and porous structure that can easily absorb and contain gases, liquids, oxidizing compounds and volatile organic molecule (Pan et al., 2018), effectively removing the volatile aldehyde and ketone.

Yeast changes the structure of the smell by increasing the aromatic compounds and decreasing the relative level of aldehyde and ketone. Tea effectively reduces the relative amount of aldehyde while increasing alkan and inhibiting the formation of trimethylamine (TMA) in the hydrolysate. Cheng Suiyang et al. (2015) also eliminated the smell in the protein hydrolysate is added with 0.05-0.2% by mass of black tea 0.5-1.0% by mass of garlic and 0.1-0.5% by mass chrysanthemum and and the mixture is heated at a temperature of 70-100°C for 30-80 minutes.

L. plantarum is normally used in food fermentation that not only increases the nutrition value but also creates aroma in the fermentation products. *L. plantarum and S. cerevisiae* fermentation has been reported to be improve the smell and increase the taste of fish hydrolysate (Pan et al., 2018). Yang Pinhong (2008) excluded the fishy smell in clam protein hydrolysate by fermenting with two strains of bacteria *Streptococcus thermophilus* and *L. bulgaricus*. Lee et al. (2016), in this study on claim protein hydrolysate, pointed out that fermentation with *S. cerevisiae* can noticeably reduce the smell. There was an agreement in the results in this study with other authors (Lee et al., 2016; Pan et al., 2018), that the highest effective deodorizing methods were using tea and bacterial fermentation with *Lactobacillus plantarum*, in which bacterial fermentation provided the highest

results and furthermore yielded more amino acid than other methods.

CONCLUSION

The different deodorizing methods provided distinctive results. The most effective procedure was using acetic acid 0.03% in combination with salt solution 1% to preprocess round scad materials and then fermented with the bacteria *L. plantarum* 1% in the round scad hydrolysis in 12 hours. The treated hydrolysate had no smell remaining, sweet taste, light golden brown color, the lowest TMA level (3.05 ± 0.38 mg/100g) and the highest amino acid level (13.76 ± 0.3 g/l).

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On the Analysis of Modifications of Genomic DNA in Endotoxemia

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ABSTRACT

Endogenous intoxication and, as a consequence, the development of multi-organ failure are extreme forms of the pathological process. The aim of the study is to look into the DNA modifications during endotoxic "pollution" of the body and to understand the mechanisms of development of dysregulation processes mediated by structural changes in DNA. The rate of endogenous intoxication was assessed by determining the generally accepted laboratory-clinical and biochemical parameters. The mononuclear cell fraction was collected by gradient centrifugation on Ficoll-Paque™ (ρ=1,077). The isolation of DNA from blood mononuclear cells was performed by using Laura-Lee Boodram. UV-spectroscopic assays of DNA solutions were carried out using a spectrophotometer UV-3600 Shimadzu (Japan). Interpretation & conclusions. The paper is concerned with the study of changes in biochemical composition and structure of genomic DNA of mononuclear cells of venous blood in patients with endogenous intoxication. The FT-IR spectra of genomic DNA of patients' venous blood mononuclear cells reveal that the band at a frequency of 1337 cm^{-1}, shifts to a higher frequency. The absorption band at 1491 cm^{-1}, becomes wider and has a more pronounced character. The absorption intensity increases at a frequency of 863 cm^{-1}, characteristic of the type of twisting in N-type sugars. The intensity of the band 1086 cm^{-1}, decreases. The corresponding alterations in the spectrum point to changes in the mutual orientation of DNA phosphate groups, resulting in changes in DNA spatial structure, and the increased proportion of DNA in the A-form. UV spectra of the DNA of the experimental samples showed a slight shift of the maximum and a small hyperchromic effect.

KEY WORDS: ENDOGENOUS INTOXICATION, DNA CONFIGURATION, DNA CONFORMATION, FOURIER INFRARED SPECTROSCOPY, UV SPECTROSCOPY, REACTIVE OXYGEN INTERMEDIATE, LIPID PEROXIDATION.

INTRODUCTION

Endogenous intoxication and, consequently, the development of multi-organ failure are extreme forms of the pathological process and require immediate and radical repairing, therefore, the study of the molecular mechanisms of the pathogenesis of endogenous intoxication syndrome is a topical issue (Savelyev et al., 2008; Sánchez-Tapia et al., 2020; Vlasov et al., 2009). Endogenous intoxication syndrome is characterized by accumulation of toxic endotoxins in tissues and biological fluids, organs damage, disruption of the functional systems of the body due to ischemia, necrotic cell death, release of microorganisms, their metabolic products and decay from the focus of invasive infection into the body, impaired excretion of endotoxins from the body and secondary toxic aggression (Koos et al., 2020).

The most important triggers for the development of the pathological process leading to the development of endogenous intoxication are free radicals, which are formed in excess in the body during various pathological processes induced by inflammation and ischemia. The involvement of new biomolecules, including proteins

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and lipids in the free radical process coupled with poor antioxidant activity in the body leads to deep destructive changes in the cells and tissues of the body, the development of dysregulation processes, which ultimately aggravates the pathological process in the body and leads to the development of multi-organ failure (Guevara-Cruz et al., 2019; Halliwell and Chirico, 1993; Valko et al., 2007). Endotoxins are the metabolites accumulating in large quantities in the tissues of the body, modified cytotoxic biomolecules, free radicals, including reactive oxygen and nitrogen intermediates having a high reaction activity (Baeuerle, 2020).

DNA, like other important structural and functional biomolecules of the body - proteins and lipids, is also exposed to various oxidative modifications, but changes in the DNA covalent structure are offset by enzymes of the body's repair system (Van Houten et al., 2018; Shimizu, 2014). However, endogenous intoxication nourishes all necessary conditions for the "long-term" presence of structural changes in the genomic DNA that can affect the DNA functional activity and the implementation of genetic processes, primarily gene transcription (Sánchez-Tapia et al., 2020). This paper presents the results of an experimental study of modifications that occur in genomic DNA of mononuclear cells in venous blood of patients with endogenous intoxication syndrome. The aim of the study is to investigate the DNA modifications during endotoxic "pollution" of the body and to understand the mechanisms of development of dysregulation processes mediated by structural changes in DNA.

MATERIAL AND METHODS

In laboratory studies were used samples of venous blood of 15 patients with endotoxicosis syndrome against the background of acute pancreatitis, after giving patient informed consent. The examined group: age – 48,3 ± 2,1 years, men – 7 (46,7%), women – 8 (53,3%). Control blood 10 samples were obtained from 15 to 60 years. Studies conducted in the period: January – May 2019. The rate of endogenous intoxication was assessed by determining the generally accepted laboratory-clinical and biochemical parameters (Karpishchenko, 2014). To determine nuclease activity isolated genomic DNA was used as the substrate of the reaction. Hydrolysis was carried out in the presence of $MgCl_2$, incubated at 37 °C during 2 hours.The mononuclear cell fraction was collected by gradient centrifugation on Ficoll-Paque™ (ρ=1,077). The received ring of mononuclear cells was taken to clean Eppendorf-type test tubes and cleansed from Ficoll impurities by phosphate-saline buffer (PBS) and concentrated in Hanks' solution. Cell viability was determined by the amount of penetration of stain into the cells (1% of trypan blue or 5% eosin solution).

The isolation of DNA from blood mononuclear cells was performed by using Laura-Lee Boodram technique (Boodram, 1999-2006). The cells were lysed with 10% of sodium dodecyl sulfate, then dispensed with 20% of proteinase K and incubated at 55°C during 2 hours. Proteins were salted out with 5,3 M NaCl solution. To deposit DNA in the supernatant cold isopropanol was used. The DNA precipitate was washed with 70% ethanol and resuspended in 0,1 M Tris-HCl, pH 8.5. UV-spectroscopic assays of DNA solutions (maximum absorption at λ260), quantitative determination of medium-mass molecules (λ280 and λ254 nm), diene conjugates in the extracted heptane-isopropanol mixture (1:1, by volume) of lipid fractions (λ232 and λ220 nm) were carried out using a spectrophotometer UV-3600 Shimadzu (Japan).

The DNA precipitate was lyophilized by freeze-drying FreeZone Plus. The DNA preparation was mixed with KBr to form pills. The IR Fourier spectra of DNA preparations were recorded on IRPrestige-21 SHIMADZU spectrometer (Japan) in the range of 400 cm^{-1}-4000 cm^{-1}. The obtained findings were statistically processed by the method of variation statistics using the Student t-test.

RESULTS AND DISCUSSION

The experiments utilized patients' blood with acute endotoxicosis, which is a complication of patients with acute pancreatitis, accompanied by significant changes in the biochemical and blood cell composition (Fig.1). The content of the average mass molecules – oligopeptides with molecular masses in the range between 500 - 5000 Da (MFM: λ280 and λ254) in patients' blood exceeded the normal values (249,2±19,08 and 296,4±22,17 relative units x 10-3, respectively) on average by 240% and 233% ($p<0.05$), which is a characteristic sign of the development of endogenous intoxication.

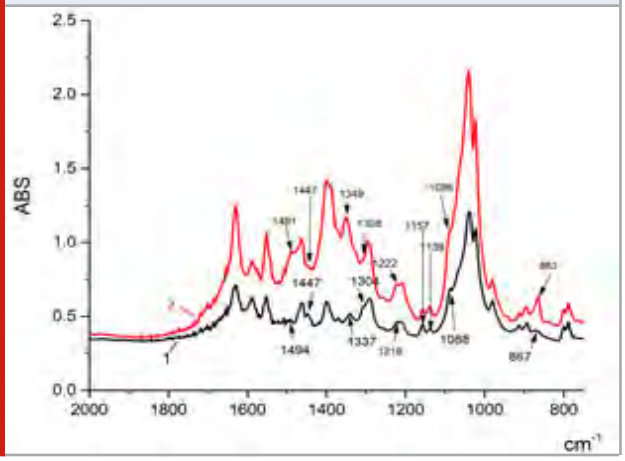
In the blood of endogenously intoxicated patients, the content of lactic and pyruvic acids was increased by 252% and 169% compared to the control samples (0,25±0,022 and 0,035±0,003 mmol/g protein, respectively) ($p<0,05$). As it was shown above in the blood of patients with endogenous intoxication, there is a more significant increase in the content of lactic acid in comparison with increased concentration of pyruvic acid, which points to the development of toxic enzymopathy. Also, blood plasma of patients with endogenous intoxication, has a high activity of DNAse equal to 240,2±2,36 activity units/l.

One of the indicators of molecular instability in the body is a high level of lipid peroxidation. In patients with endogenous intoxication syndrome, the content of the primary products of lipid peroxidation – diene conjugates (DC) on average exceeded the normal values (0,45±0,01 rel.units/mg. of lipids) by 3,3 times, and TBA-reactive substances, which include malon dialdehyde, exceeded the control parameters (4,5±0,5 nmol/ml) by an average of 220% ($p<0,05$). To study the properties of genomic DNA, we utilized the blood of endogenously intoxicated patients whose core biochemical parameters (markers) were higher or lower than the corresponding normal values. By using spectral methods of analysis in IR and UV regions of the spectrum we studied structural modifications of genomic DNA (changes in chemical

structure, conformations, packing density) in endogenous intoxication.

Analysis of absorption spectra of control and experimental samples in the IR region (Fig. 1) showed the presence of DNA-specific bands in the frequency range 2960 - 2850 cm^{-1}(A), 1500 - 1250 cm^{-1} (B), and 1150 - 750 cm^{-1} (C), which are usually attributed to the bands caused by vibrations in the bonds of CH2-groups, bonds between the bases and sugars (the fluctuation region of the groups in the sugar-phosphate backbone of DNA) and symmetrical vibrations in the bonds of phosphate groups $-PO_2$, respectively (Stuart 2004; Zhizhina and Oleynik, 1972). At the same time, the IR spectra of the genomic DNA in the control and experimental samples differed by the intensity of absorption of characteristic bands and the shift of absorption bands.

Figure 1: IR-spectra changes in DNA of donors' blood (1) as opposed to patients with endogenous intoxication syndrome (2)

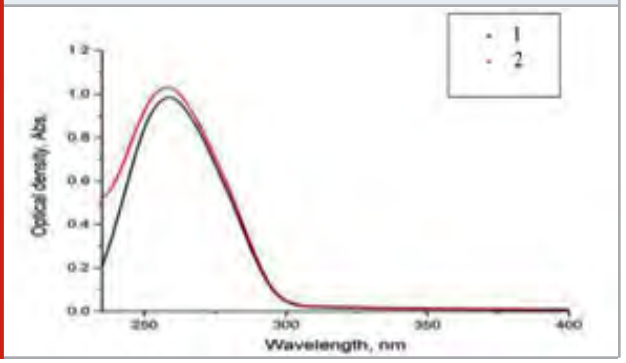


Of particularly note are changes in the IR-spectrum of the DNA blood of patients with the syndrome of endogenous intoxication in the areas 1500-1250 cm-1 and 1150-750 cm^{-1}, as they may indicate rearrangements in the sugar phosphate backbone of the DNA, associated in particular with changes in efficiency of the ionic interactions between the functional groups of separate molecular fragments, which maintain a specific DNA conformation (Spirin, 1958; Taillander et al., 1985). As it can be seen from Fig. 1, experimental samples, as opposed to control samples, in the range between 1500-1250 cm^{-1}, have a decreased absorption intensity in the region 1447 cm^{-1} and 1308 cm^{-1}, an increased intensity and shifting of the band at a frequency of 1337 cm^{-1}, characterizing the vibrations of -CH2 group, the vibrations of deoxyribose-nitrogen base bonds, glycoside dihedral angles into the region of higher frequencies - 1349 cm^{-1}. The absorption band of genomic DNA of patients with endogenous intoxication 1491 cm^{-1}, reflecting the movements of purine atoms and the corresponding C=N guanine bond (Polyanichko et al., 2004; Eichhorn and Shin, 1968), is getting wider and has a more obvious character.

In the region 1150-800 cm^{-1}, where we have vibrations of the sugar-phosphate backbone, noticeable shifts of the absorption bands are not observed. At the same time, the absorption intensity caused by symmetric vibrations of the bonds in the group O=P=O at frequencies 1086 cm^{-1}, 1157 cm-1 and 1222 cm^{-1} decreases, and increases at a frequency of 1139 cm^{-1} and the absorption intensity at a frequency of 863 cm^{-1}, characteristic of n-type sugars increases significantly. It is known that the spatial structure of DNA is polymorphic, that is, it can take different conformations, including three main types of structures – A-, B- and Z- forms. For each of these forms in the IR spectra there are so-called marker absorption bands, which can tell us about the geometry of the DNA macromolecule, the possible transitions between conformations, the stability of the structure (Zhizhina and Oleynik, 1972; Stuart, 2004; Taillandier and Liquier, 1992).

Normally, the DNA isolated from the control samples is mainly represented by the B-form: absorption bands are observed at frequencies of 970, 896 and 837 cm^{-1}, characteristic of DNA in the B-form (Spirin, 1958) and partially in the A-form – absorption band at a frequency of 863 cm^{-1} (Taillandier and Liquier, 1992; Tsuboi, 1969).

Figure 2: UV spectra of DNA from donors' blood (1) against patients with the syndrome of endogenous intoxication (2)



As it was shown above, in DNA, isolated from samples, the band shifts at a frequency of 1337 cm^{-1} (vibrations of methylene radicals) to the region of higher frequencies - 1349 cm^{-1}, as well as there is a noticeable increase in the absorption intensity at a frequency of 863 cm^{-1}, (absorption band regular of the type of twisting in n-type sugars -C3'-endo/anti, 870-865 cm^{-1}, marker of A-form) (Stuart, 2004). In addition, the intensity of the 1086 cm^{-1} band related to the symmetric vibrations of the bonds of O=P=O backbone decreases (Spirin, 1958; Taillander et al., 1985). Such changes in the spectrum point to changes in the mutual orientation of the DNA phosphate groups, which is a consequence of the local unwinding of the double helix, the redistribution of hydrogen bonds between the nitrogen bases, the appearance of bends in it, resulting cumulatively in a change in the spatial structure of DNA.

In genomic DNA, isolated from the blood of patients with endogenous intoxication syndrome, most likely, there is a relative increase in the proportion of DNA in the A-form. According to the literature, under physiological conditions (low salt concentration, high degree of hydration), the dominant structural type of DNA is a B-form. An A-form is formed under conditions of lesser hydration and a higher ion content. Apparently, during evident endotoxicosis, a complex of conditions is formed under which DNA modification occurs, consisting in structural changes and conformational rearrangements.

One of the methods of detecting changes in the structure of DNA molecules is the record of absorption spectra in the UV region. Firstly, by absorption ratio at wavelengths of 260 nm and 280 nm (λ260/ λ280) one can estimate the purity of the DNA preparation. The preparation is considered pure if the ratio of 260 nm/280 nm is approximately 1,8 for DNA. Secondly, by changing the nature of absorption at a wavelength of 260 nm, one can judge the stability of the bonds between complementary pairs, ensuring the maintenance of the necessary DNA conformation (Doshi et al., 2009). Intensive absorption of nucleic acids in the UV region of the spectrum is caused by purine and pyrimidine bases. Absorption spectra of select bases merge into one wide band and give a characteristic absorption of DNA with a maximum absorption at λ= 260 nm. Our studies have shown that the UV spectra of genomic DNA isolated from donors' blood and patients with endogenous intoxication syndrome are different (Fig 2).

Figure 2 shows that in the UV absorption spectra of DNA, the absorption ratio at wavelengths of 260 nm and 280 nm of control and experimental samples lies within 1,8, which indicates a sufficiently high purity of the isolated macromolecules. At the same time, in the DNA spectra of the experimental samples there is a relative displacement of the maximum and a slight hyperchromic effect.

Quantum-mechanical calculations show that the intense absorption of light by purine and pyrimidine bases at 260 nm is linked with p-p and to a certain extent with n-p transitions. The optical properties of chromophore groups depend on the conformation of DNA. The phenomenon associated with an increased optical density is called the hyperchromic effect, and vice versa, the reduced absorption of native DNA preparations - hypochromic effect. Such changes in the spectra may also be attributed to changes in the DNA structure of blood cells in patients with endogenous intoxication syndrome. We would like to emphasize that DNA spatial structure disorders can be the result of accumulation (penetration) of products, including those of toxic ones, in the nucleic acid microenvironment, formed because of impaired balance of biochemical reactions during intoxication. In its turn, changes in the DNA conformation can be the cause of disruption of genetic processes, including gene expression and, consequently, lead to the development of dysregulation processes in the body of patients.

During various inflammatory conditions, one of the undesirable stages of the disease is the development of endogenous intoxication syndrome and, consequently, the development of multi-organ failure.During endogenous intoxication, toxic endotoxins accumulate in the body, causing aggravation of the patient's condition and the formation of this condition, that requires timely diagnosis and immediate repair. According to the obtained findings during the development of endogenous intoxication syndrome in patients' blood cells there occur changes in the structure of genomic DNA. The changes in the genomic DNA spatial structure in the syndrome of endogenous intoxication can also be revealed through changes in the UV spectra of DNA samples, observed as a slight shift of the maximum and a small hyperchromic effect.

The observed changes in the UV spectrum of the DNA medicine isolated from the blood of patients with endogenous intoxication syndrome are usually attributed to the binding of metal ions to the DNA molecule at N7 guanine position (Kasyanenko et al., 1989; Koos et al., 2020). Such binding leads to destabilization of hydrogen bonds between complementary pairs, and with a significant number of such binding sites, there is a disruption of base-stacking, accompanied by a hyperchromic effect in the DNA absorption spectrum (Kasyanenko et al., 2014; Sánchez-Tapia et al., 2020). Several papers have shown (Doshi et al., 2009; Khan Asia et al., 2006; Doshi et al., 2010) that the saturation of DNA solutions with oxygen leads to an increased absorption with a maximum at λ260.

Such hyperchromism is attributed to one-electron oxidation of DNA by singlet oxygen (Baeuerle, 2020; Kanvah et al., 2009) and the formation of its complex with superoxide anion, DNA+•O2–, by a product of singlet oxygen reduction.It is known that singlet molecular oxygen initiates some biological processes that lead to oxidative stress, accompanied by hyperproduction of reactive oxygen species, which can damage nucleic acid molecules. DNA is one of the main targets for reactive oxygen species, which have genotoxicity and lead to mutations. Modification of DNA effectuated by singlet oxygen is expressed almost exclusively as oxidation of purines. Whereas OH-radical, effectively interacting with deoxyribose, purine and pyrimidine bases leads to more serious changes in the DNA structure. Oxidized purines and pyrimidines are products of the induced damages that are very common in DNA under oxidative stress (Kanvah et al., 2009; Kim et al., 2020). It was noted above that during endogenous intoxication syndrome, the concentration of lipid peroxidation products (LPP) increases in patients' blood, and accumulation of acids creates prerequisites for reducing the pH values.

The initiators of LPO are reactive oxygen intermediates. In particular, singlet oxygen can be formed following the interaction of hydroxyl radicals with superoxide anion radicals, which in turn can be formed as a response to high concentrations of ions, for instance K+, and

contribute to the development of a vicious circle of free-radical modification of biomolecules, cells and tissues damage.During lipid peroxidation, a variety of cytotoxic products accumulates, including aldehydes. In particular, malondialdehyde (MDA) is formed as a result of peroxidation of fatty acids containing three or more double bonds (linolenic and arachidonic acids, respectively). The reaction of MDA with primary amines leads to the formation of Schiff bases. MDA can bind to the DNA nitrogenous bases, leading to a cross-linking of DNA chains, therefore MDA is assigned to the role of a mutagenic, genotoxic, and carcinogenic compound (Freeman and Crapo, 1984Uzbekov, 2014; Guevara-Cruz et al. 2019).

Earlier we have shown that under conditions of increased activity of lipid peroxidation, cells respond by modification of chromatin lipids, coupled with changes in the amount of separate lipids and degree of their oxidation (Trofimov et al., 2013 Sánchez-Tapia et al., 2020). Therefore, on the basis of research findings and data of scientific literature it can be assumed that in severe forms of inflammatory diseases accompanied by endogenous intoxication, hyperproduction of reactive oxygen species, intensification of free radical processes, including lipid peroxidation, impairs the barrier and regulatory properties of the nucleus membranes, resulting in penetration of excess ions and other strange to karyoplasm substances penetrate karyoplasm, local changes in pH of the medium and in degree of hydration (Kim et al., 2020).

The interaction of reactive oxygen species with DNA, most likely and primarily with the nitrogen guanine bases, leads to modification of the macromolecule structure and conformational rearrangements: as evidenced by the change in the vibrations of methylene groups, vibrations in the sugar-base bond groups, changes in glycosidic dihedral angles (band shift at a frequency of 1337 cm^{-1} into the region of higher frequencies – 1349 cm^{-1}), changes in vibrations of the C=N guanine bond (shift and increase in the absorption band at 1491 cm^{-1}, reflecting the movements of purine atoms) (Polyanichko et al., 2004; Eichhorn and Shin, 1968). These changes to a certain degree are provoked by interaction of lipid peroxidation products with nitrogen bases (Baeuerle, 2020).

CONCLUSION

The paper discusses the analysis of improvements in the biochemical composition and structure of the genomic DNA of venous blood mononuclear cells in patients with endogenous poisoning. The FT-IR genomic DNA spectra of the venous blood mononuclear cells of patients show that the band shifts to a higher frequency at a frequency of 1337 cm^{-1}, characterizing the fluctuations of the CH2 group and sugar-base bonds. The absorption band at 1491 cm^{-1}, reflecting the movement of purine atoms and corresponding to –C=N guanine bond, becomes wider and has a more pronounced character. The absorption intensity increases at a frequency of 863 cm^{-1}, characteristic of the type of twisting in N-type sugars. The intensity of the band 1086 cm^{-1}, capturing the symmetric vibrations of the O=P=O stroma bonds decreases.

The related spectrum changes reflect changes in the reciprocal orientation of the phosphate groups of DNAs arising from the topical untwisting of the double helix, the redistribution of the hydrogen bonds, the presence of bends on the helix resulting in changes in the spatial structure of DNA, and the increased proportion of DNA in the A-form. The DNA UV spectra of the experimental samples demonstrated a slight maximum shift and a minor hyperchromic effect. The observed changes in the spatial structure of DNA may be due to the accumulation (penetration) of microenvironmental DNA materials, including toxic ones, resulting from impaired biochemical reaction equilibrium during intoxication. In turn, variations in DNA conformation may be the cause of genetic process disorders, including gene expression disorders, leading to the creation of dysregulation processes in the bodies of patients.

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Evaluation of Soft Diet Served in Five Private Hospitals in Amman, Jordan

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ABSTRACT

Meals of soft diet was evaluated, in five private hospitals located in Amman, Jordan. Menus of the diets were analyzed by calculating their content of energy, fiber, macronutrients and some micronutrients, (vitamins and minerals) then compared with standards. The mean content of energy insoft diet was 1775 kcal and the mean content of dietary fiber was 33.4 g. Carbohydrate, protein and fat contribution of the total energy were within the acceptable macronutrients distribution range in the diet. Soft diet contains excessive levels of sodium which exceeded the upper limits (UL) of 2300 mg. Overall, hospital meals provided a diet low in the following nutrients (potassium, vitamin D, vitamin E and vitamin K) and did not meet the nutritional standards. Based on "Choose My Plate" recommendations, mean contents of food groups of the three diets were ranged as follows: grains, 3.2-4.6 serving/d; protein, 2.6-3.4 serving/d, vegetables, 4.9-5.5 serving/d, fruits, 1.2-1.5 serving/d and dairy 3.1-3.4 serving/d. Many hospitals do not design diets to meet dietary recommendations. Hospital menus should be continuously evaluated to reflect the changes of the patients' needs.

KEY WORDS: SOFT DIET, HOSPITAL DIETS , MACRONUTRIENTS, MICRONUTRIENTS, JORDAN .

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INTRODUCTION

Adequate nutrient supply, covering energy, macro- and micronutrients is very important for good health and well-being. Inadequate nutrient supply can lead to malnutrition, whereas excessive nutrient supply may cause various diet-related diseases, such as obesity, diabetes, or cardiovascular diseases (Grochowska-Niedworok et al. 2019). A healthy diet is the one that guarantee regular intake of necessary nutrients according to individual needs. In hospitals, a healthy diet served for patients is the one that take into consideration individual needs of necessary nutrients according to their health conditions (Dumlu et al. 2014 ; The Scottish Government 2008). In patients with infectious diseases, good nutrition has positive effects, whereas, poor nutrition may slow down recovery from infectious disease due to the adverse effects on host immune function (AslıGizemPekmezci et al. 2018 ; Heidegger et al. 2013 ; Gianotti et al. 2002).Low intake of protein and energy leads to adipose tissue atrophy, immune deficiency, muscle wasting, poor digestion and inhibited nutrient absorption. To ensure optimal health, it is vital to consume a well-balanced diet containing adequate protein supply (Grochowska-Niedworok et al. 2019).

Nutritional therapy is an essential component of the management of disease and has an important role in helping achieve and maintain optimal control (Department Of Social Services, 2020). Generally, all hospital diets should meet the nutritional requirements of the assisted patient. Studies suggest that dietary recommendations are not met among hospitalized patients (Patricia et al. 2004 ; Wright et al. 2004). In our literature search, we found few published papers describing hospital patient menus (Al-Domi et al. 2011 ; Ahmad 2014 El-Qudah 2018) and therapeutic diets in Jordanian hospitals (Shaheen 1998 ; Bawadi and Abu-Jamous 2014).Thus, this study was conducted in five private hospitals located in Amman, Jordan to evaluate the mean content of soft dietof macronutrients, some micronutrients and energy and number of food groups based on my plate guide of meals served at these hospitals.

MATERIAL AND METHODS

This study is an observational one with a cross- sectional design. It was adopted to determine the content of one therapeutic diet (soft diet) of energy and some nutrients. The 3-day meal plans provided by each hospital were evaluated,in five hospitals, chosen randomly, located in Amman , Jordan. The data were collected over a period of three months (September to December 2014). The required permit to conduct the study was obtained from the Committee of Scientific Research. The study was approved by the department research committee, Nutrition and Food Processing Department at Al-Balqa Applied University. In addition, administrative approval were sought from the authorities on each hospital.

We visited nutrition department in each hospital for interviewing the dieticians in charge. We record menus of three days soft diet. Amounts of ingredients for recipes in every item served as the prescribed meal plans for each hospital were recorded in a comprehensive database that allowed precise nutritional analyses by weight of food serving. The composition of the diet was analyzed in terms of the nutrient and energy content based on the United States Department of Agriculture Database, super tracker (United States Department of Agriculture. Super Tracker. Access on 2017) and food exchange system. The mean of three day nutrient contents of meals (2 weekdays and 1 weekend day) was calculated. Macro nutrients (carbohydrates, protein and fat), energy, dietary fiber and certain micronutrients are recorded for each food item.

The total values of each nutrient/day are summed&t then an average is made upon the three days nutrient sums. Nutrient levels in soft diet menus were compared with standards based on the USDA's 2010 Dietary Guidelines for Americans (requirements for adult populations) (IOM Institute of Medicine, 1997/2005 and 2011 ; The Dietary Guidelines for Americans, 2010 ; New York City Food Standards: Patient Meals, 2012; Food Standards: Patient meals 2012). Recommended total daily amounts of food groups of the Choose My Plate guidelines were used for assessment of contents of these meals in each hospital.

Statistical analysis: Data analysis was performed in SPSS version 21. Descriptive statistics were usedand the data were expressed as mean, SD, frequency and percentage.

RESULTS AND DISCUSSION

The Mean daily contents of food groups were above the recommendations of dairy and vegetables, while grains, fruits and protein groups were below the recommendation in soft diets served by hospitals (Table 1).

Table 1. Mean content of serving number of food groups of soft diet, served in five Hospitals

Food groups	Serving number	Goal (serving/d)
Grains (oz)	3.8	6
Proteins (oz)	3.2	5
Vegetables (cups)	4.9	2.5
Fruits (cups)	1.3	2
Dairy (cups)	3.1	3

The mean daily energy content of the soft diet was 1775 kcal. The mean daily content of dietary fiber in the soft diet was 33.4 g, which is above the recommendations (Table 2). As seen in Table (3), CHO, protein and fat contribution of the total energy in the diet was within the AMDR. Overall, the mean content of minerals in the diet was within the standards (Table 4).As shown in (Table 5), the mean content of all vitamins in the soft diet, meet the nutritional standards, with the except of vitamin B6, B12 and niacin which were above the recommendations.The mean content of vitamin E and

vitamin D was below the recommendations, while it was within the recommendation for vitamin A and vitamin K in the soft diet (Table 6). Based on the results of the present study, it seems that soft diet generally contain adequate nutrients.

Table 2. Mean content of macronutrients, Dietary Fiber, cholesterol and energy

Type of diet	Macronutrients			Energy(kcal)	Dietary Fiber (g)	Cholesterol (mg)
	CHO (g)	Protein (g)	Fat (g)			
Soft	231	91	54.1	1775	33.4	259.3

Table 3. Macronutrients percentage contribution of the total energy

Type of diet	CHO (%)	Protein (%)	Fat (%)
Soft	52.1	20.5	27.4

Table 4. Mean content of micronutrients in five private hospitals in Amman

Type of diet	Calcium (mg)	Potassium (mg)	Sodium (mg)	Iron (mg)	Magnesium (mg)	Phosphorus (mg)	Selenium (µg)	Zinc (mg)
soft	1018	3112	3307	29.6	255.9	1381	110.9	10.7

Table 5. Mean content of water soluble vitamins in five private hospitals in Amman

Type of diet	Vitamin C (mg)	Vitamin B6 (mg)	Vitamin B12 (µg)	Folate (µg)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Choline (mg)
soft	97.3	2.07	3.947	430.8	1.325	1.922	23	328.7

Table 5. Mean content of water soluble vitamins in five private hospitals in Amman

Type of diet	Vitamin A (µg)	Vitamin E (mg)	Vitamin D (µg)	Vitamin K (µg)
Soft	714.9	6.9	1.9	72.5

The evaluation of 3-day meal plans offered in the five private hospitals showed the mean daily content of calorie, macronutrients and many micronutrients were adequate and within recommendations, with the exception of some nutrients. Similar studies support these findings (El-Qudah 2018 ; El-Qudah 2016 ; Kyungjoo et al. 2010) . The analysis menus showed that the average amount of calcium supplied from soft diet was sufficient. Similar results were found in other studies (Shaheen 1998 ; El-Kadiki and Sutton 2005 ; Fulgoni et al. 2007).The results showed that hospital meals provided a diet low in vitamin E and vitamin D in the soft diet. Both nutrients did not meet the nutritional standards. Similar results were obtained by other studies (Moreira et al. 2012 ; Franklin et al. 2004). In a study conducted in Jordan, meals of regular diet were evaluated, in seven Governmental hospitals located in three cities in the middle region of Jordan (El-Qudah 2018). In this study, all hospital diets provided low content of calcium, vitamin D and vitamin E and did not meet the recommendations (El-Qudah 2018). Although, hospitalized patients should receive a standard diets suitable for their diseases, there have been numerous reports that the nutritional intake of many hospitalized patients is suboptimal.

CONCLUSION

This study showed that many hospitals do not design diets to meet dietary recommendations. Hospital menus should be continuously evaluated to reflect the changes of the patients' needs.

Study Limitations: Hospitals included in this study were limited to the private hospitals, therefore, are not representative of all hospitals in . In addition,the results should be interpreted with caution given that we did not analyze meals directly.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Does the Perception of Self-Ligating Brackets Differ from Conventional Brackets Among Orthodontists?

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ABSTRACT

The purpose of this study was to determine orthodontists' perception of the reported advantages of self-ligating brackets (SLB) during their daily practice and if there is a relationship between their bracket preference and the advantages of that bracket system. An online survey was distributed to 173 orthodontists and orthodontic residents to compare their perceptions of SLB and CB (conventional brackets). The questionnaire consisted of 2 sections: Section A was composed of questions of the individual practitioner's characteristics and experience with SLB; Section B assessed a variety of treatment factors, allowing orthodontists to indicate a preference for either SLB or CB based on their experience and perceived clinical results. Orthodontists preferred SLB in initial treatment and shorter adjustment appointments (P<0.0001). CB were preferred for cost, space closure, finishing, and detailing (P<0.0001). Participating orthodontists perceived a clinical difference between SLB and CB. Orthodontists preferred SLB in 7 out of the 12 treatment factors that were evaluated. SLB were preferred mostly for initial treatment progress, shorter adjustment appointments, oral hygiene, and less required extraction. CB were preferred mostly for space closure, finishing, and cost-effectiveness according to the respondents.

KEY WORDS: SELF-LIGATING BRACKETS, PERCEPTION, PREFERENCE, CONVENTIONAL BRACKETS.

INTRODUCTION

Self-ligating brackets (SLB) are a bracket system with no ligatures needed to secure the archwire in the bracket slot (Cacciafesta et al., 2003). The system has a built-in mechanical device to close the bracket slot (Cacciafesta et al., 2003). The engagement between the bracket slot and the archwire is produced by a metal labial door (passive SLB) or by a clip (active SLB), both replacing the steel ligatures and the elastomeric modules (Fleming et al., 2008). There are a number of claimed advantages of SLB over the conventional bracket (CB) – the most important is reduced friction between the bracket slot and the archwire, which results in faster tooth movement (Harradine, 2003; Harradine, 2008; Sfondrini et al., 2018). Also, since it produces less incisor proclination and greater expansion, less extraction is required to relieve crowding, (Birnie and Harradine, 2008). Other reported advantages were improved treatment efficiency, less need for assistance during adjustments, shorter overall

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treatment time, better patient comfort, oral hygiene, and increased patient adherence and acceptance (Rinchuse and Miles, 2007; Harradine, 2008; Prettyman et al., 2012 Sfondrini et al., 2018; Rathi et al., 2019).

The literature provides conflicting data regarding the treatment efficiency and friction of SLB compared to CB (Harradine and Birnie 1996; Harradine, 2001; Miles et al., 2006; Prettyman et al., 2012). Some studies reported less friction with SLB (Pizzoni et al.,1998; Miles, 2005; Cordasco et al., 2009), while others reported that when angulation and tipping are accounted for, these brackets produce similar or higher friction compared to CB (Bednar et al., 1991; Redlich at al., 2003). Ehsani et al. reported in their systematic review that less friction is produced when small round archwires are used with SLB compared to CB (Ehsansi at al., 2009). However, no sufficient evidence was found when larger rectangular archwires were used in maligned arch (Ehsansi at al., 2009).

Several studies on treatment efficiency reported a shorter treatment duration (4-6 months less) and fewer appointments for patients treated with SLB compared to patients treated with CB (Eberting et al., 2001; Turnbull and Birnie, 2007). On the other hand, several studies reported no difference in the total treatment time between SLB and CB (Pandis et al., 2006, Turnbull and Birnie, 2007; Yorita, 2007). Systematic reviews on the efficacy and of SLB reported a lack of evidence for the advantages of SLB over CB (Pandis et al., 2007; Fleming and Johal, 2010; Yang et al., 2017). Consequently, the popularity of SLB might be caused by effective marketing. The purpose of this study was to determine the perception of the orthodontists on the reported advantages of SLB during their daily practice and if there is a relationship between their bracket preference and the advantages of that bracket system.

MATERIAL AND METHODS

Before beginning the study, the study protocol was approved by the Institutional Review Board (18/0407/IRB) of King Saud University, College of Medicine in Riyadh, Saudi Arabia. The questionnaire used in this study was taken from a previous study by Prettyman et al., which was composed of a cover page including the title and purpose of the research project (which clearly explained the utility of participating). The questions consist of two sections: Section A was composed of questions to obtain individual practitioner characteristics and focused on the responding clinician's experience with SLB; section B assessed a variety of treatment factors, allowing orthodontists to indicate a preference for either SLB or CB based on their experience and perceived clinical results (Prettyman et al., 2012).

Each question had a blank section for respondent comments.Assuming 70% of preference for SLB by orthodontists with ± 7% precision and at a 0.05% level of significance, 165 orthodontists were the effective sample required. Due to the nature of an online survey, only 30 to 40 % of responses were expected, hence the enhanced number of orthodontists and orthodontic residents (500 registered in the Saudi Orthodontic society) targeted by email to participate in this study.

Data were analyzed using SPSS version 24.0 (IBM Inc., Chicago, USA) statistical software. Descriptive statistics (frequencies, percentages, and mean) were used to describe the categorical and quantitative variables. Pearson's Chi-square test was used to compare the distribution of responses across the categorical variables and to assess the association between the responses of categorical variables. Student's t-test for single sample was used to compare the mean preference scores with null value of zero. A p-value of ≤ 0.05 was used to report the statistical significance of findings.

Table 1. Distribution of characteristics of study subjects(n=173)

Characteristics	No.(%)
Gender	
Male	78(45.1)
Female	95(54.9)
Position	
Postgraduate student	38(22)
Specialist	50(28.9)
Consultant	59(34.1)
Academician	26(15)
No. of years practice in orthodontics	
Less than 2 years	19(11)
2-5 years	57(32.9)
6-10 years	39(22.5)
More than 10 years	58(33.5)
Qualification	
Masters	55(31.8)
Board	85(49.1)
PhD	33(19.1)
Place of study for an orthodontist	
National program	93(53.8)
USA	34(19.7)
Europe	26(15)
Other places	20(11.5)
Working place	
Educational institute	66(38.2)
Governmental hospital	71(41.0)
Private clinic.	36(20.8)
Have you used Self-ligating brackets?	
Yes	121(69.9)
No	52(30.1)

RESULTS AND DISCUSSION

Out of 500 survey invitations, 173 orthodontists completed the survey. 54.9% were female, about 63% were specialists and consultants, 15% were academics, and 22% were postgraduate students. More than 50% of them had 6 years of practice (or more) in orthodontics.

31.8 % of study subjects held master's degrees, had board qualifications (49.1%), or completed a PhD program (19.1%). 53.8% of subjects studied in a national program and the remainder studied outside the Kingdom. About 80% of them were working in educational institutes and government hospitals. As part of treatment in their practice 69.9% (121 orthodontists) responded positively that they were using SLB. (Table 1).

A high proportion (86.8%) of orthodontists were currently treating up to 30 % of their patients with SLB, which is statistically significant (p<0.0001). About 52.9% of them stated that they had been using SLB for less than 2 years and 10.7% were using it for more than 10 years, which was statistically significant (p<0.0001). A statistically significant proportion (63.6%) of orthodontists mentioned that they considered fewer than 10 cases before they were accustomed to SLB and felt comfortable using this technique (p<0.0001). Using SLB as a marketing tool in their practice was denied by 81%, which is highly statistically significant (p<0.0001). The average appointment intervals for CB was 4 to 5 weeks in 90.9% of the study subjects, which is also highly statistically significant (p<0.0001). 44.6% and 42.1% reported that their average appointment intervals for SLB were 4 to 5 weeks and 6 to 7 weeks respectively (p<0.0001, see Table 2).

Table 2. Distribution of Practicing Characteristics of study subjects(n=121)

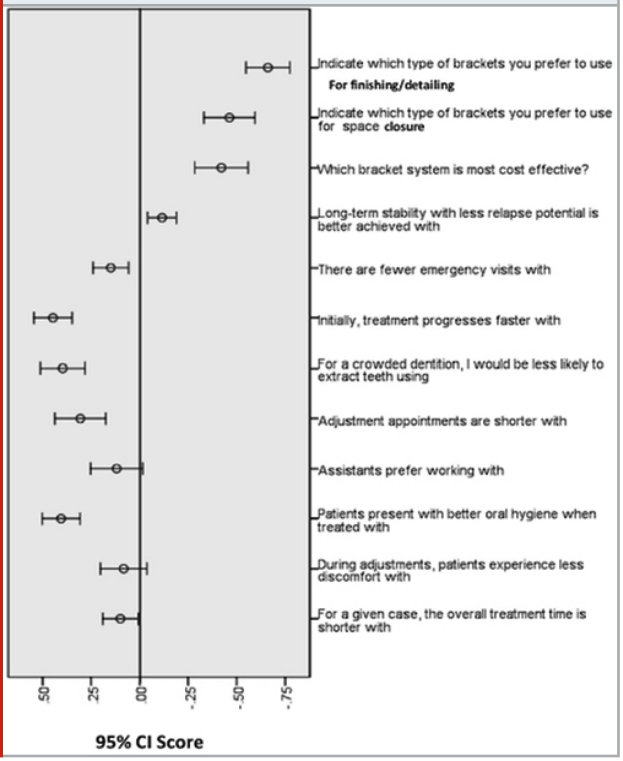
Characteristics	No.(%)	χ^2-value	p-value
Approximately what % of your patients do you currently treat with self-ligating brackets?			
0 to 30	105(86.8)	155.97	<0.0001
31 to 70	11(9.1)		
71 to 100	5(4.1)		
How long have/had you been using self-ligating brackets?			
Less than 2 years	64(52.9)	32.74	<0.0001
2 to 10 years	44(36.4)		
More than 10 years	13(10.7)		
How many cases did it take for you to become accustomed to self-ligation and feel comfortable using this technique?			
Less than 10	77(63.6)	101.97	<0.0001
10 to 30	20(16.5)		
More than 30	4(3.3)		
Never became comfortable	20(16.5)		
Do/did you use self-ligating brackets as a marketing tool for your practice? Yes	23(19)	46.49	<0.0001
No	98(81)		
What are/were your average appointment intervals for conventional brackets? 4 to 5 weeks	110(90.9)	247.91	<0.0001
6 to 7 weeks	9(7.4)		
8 to 9 weeks	1(0.8)		
10 or more weeks	1(0.8)		
What are/were your average appointment intervals for self-ligating brackets?			
4 to 5 weeks	54(44.6)	67.99	<0.0001
6 to 7 weeks	51(42.1)		
8 to 9 weeks	14(11.6)		
10 or more weeks	2(1.7)		

Figure 1 shows the orthodontists' preference for either SLB or CB. The bracket preferences are shown as 95% confidence. A preference scoring of -1 was used for CB, 0 for no difference, and +1 for SLB.

The responses of 12 treatment factors towards the type of bracket preferences (SLB, CB or no preferences) among orthodontists were assessed. A preference scoring of -1 was used for CB, 0 for no difference, and +1 for SLB.

The statistical test for a significant difference means preference with its p-value indicates a preference for either SLB or CB (Table 3). Regarding treatment time, 18.2% reported that treatment time was shorter with SLB, 8.3% reported that CB was shorter, and 73.6% reported no difference in the overall treatment time between the two types. Thirty-two orthodontists (26.2%) mentioned that their patients experienced less discomfort with SLB, 22 (18.2%) indicated the same for CB, and 67 (55.4) reported no difference. More than half of the orthodontists (54.5%) reported that there was no difference in the oral hygiene of their patients treated with SLB or CB, 43% reported better oral hygiene with SLB, and only 2.5% reported better oral hygiene with CB (P<0.0001).

Figure 1: Preferences for SLB or CB for a variety of treatment factors. The bracket preferences are shown as 95% confidence. (A preferences scoring of -1 was used for Cb, 0 for no differences and +1 for SLB. An average score of zero indicate no prefernces



Forty eight percent of orthodontists indicated shorter adjustment appointments, less likelihood of extraction (44.6%), and faster progress of initial treatment with SLB (54.5%; P<0.0001). Assistants preferred working with SLB (P<0.046). When asked which type of bracket was associated with fewer emergency visits, 71.9 % reported no difference, 21.5% mentioned SLB, and 6.6% indicated CB (P<0.002). CB is more cost-effective according to the orthodontists (58.7%) and they prefer it for space closure (60.3%) and finishing (74.4%) (P<0.0001). Although 81.8% of our sample stated that there was no difference in stability between the two types of brackets, 14.9% stated that cases treated with CB were more stable and 3.3% preferred SLB for stability (P<0.003; Table 3).

In orthodontic practice, SLB are used in high or low percentages according to practitioner preferences. Our results showed that 86.8% of respondents treated less than 30 % of their patients using SLB while 4.1% reported that they are using them in treating 70%-100% of their cases. These findings were similar to the previous study by Prettyman et al. who reported that 52% of their sample used SLB in fewer than 30% of their patients and 33% reported to use them for most patients (Prettyman et al., 2012). More than half of respondents (52.9%) reported that they had been using SLB less than 2 years, which did not align with the previous study where 73% of orthodontists reported that they were using SLB for 2-10 years (Prettyman et al., 2012), which might be due to the difference in sample size, or it might reveal that SLB became more popular recently in Saudi Arabia. Most of the respondent denied being using SLB as a marketing tool and 42.1% reported average appointment intervals of 6-7 weeks, which agreed with the previous study (Prettyman et al., 2012).

The overall treatment time was found lower for SLB (P<0.033); however, 73.6% stated no difference between the two systems which agreed with Prettyman et al. (Prettyman et al., 2012). Nevertheless, 54.5% of the orthodontists reported faster initial treatment progression with SLB, which was also reported in previous studies (Prettyman et al., 2012; Rathi et al., 2019). Treatment efficiency using SLB has contradicting results in the literature: some studies reported that patients treated with SLB finish treatment 4-6 months earlier than those treated with CB (Eberting et al., 2001; Harradine, 2001; Harradine, 2013) while others reported few differences in terms of treatment efficiency and space closure between the two systems (Fleming and Johal, 2010; Johansson and Lundstorm, 2012). Patients' discomfort was insignificant (P=0.175), which agreed with previous studies (Prettyman et al., 2012; Yang et al, 2017).

Oral hygiene among orthodontic patients treated with SLB was better due to the absence of elastomeric modules which cause plaque retention (Pandis et al, 2008). In our study 43% reported better oral hygiene with SLB while 54% indicated no difference (P<0.001). This agreed with previous studies where orthodontists indicated a significant preference for SLB with regards to oral hygiene (Prettyman et al., 2012; Rathi et al., 2019). However, different studies found that there was no statistical difference in oral hygiene between patients treated with SLB and those treated with CB (Henao and Kusy, 2004; Burrow, 2009; Ehsani et al., 2009; Al-Anezi, 2014; Yang et al., 2017).

A systematic review by Chen et al. found that shortened chair time and less incisor proclination were the only significant advantages of SLB (Chen et al., 2010). In our study, 47.9 % (P<0.0001) reported shorter adjustment appointments with SLB. The same finding was reported by a previous study with 64% of their sample (P<0.0001) (Prettyman et al., 2012). Since SLB produce less incisor proclination and more expansion in the dental arch posteriorly, it has been claimed that their cases require

less extraction (Birnie, 2008; Chen et al, 2010). In our study, 54.5 % of orthodontists reported no difference between the two types of brackets on extraction decisions, which was consistent with previous studies addressing extraction decisions, intercanine width, and incisor proclination (Pndis et al., 2009; Fleming et al., 2009; Prettyman et al., 2012; Jacobs et al., 2014).While our sample preferred SLB for initial alignment, they preferred CB for space closure (60.3%, P<0.0001). Burrow et al. reported faster canine retraction with CB than SLB (Burrow, 2010). Other studies found no differences in space closure between the two types (Miles, 2007; Prettyman et al., 2012; Songra et al., 2014).

Table 3. Association between treatment factors Responses and Bracket Preference among Orthodontists

Treatment Factor	Responses -No.(%)			Mean Preference Score (P-Value)
	SL	CB	No difference	
For a given case, the overall treatment time is shorter with	22(18.2)	10(8.3)	89(73.6)	0.10(0.033)
During adjustments, patients experience less discomfort with	32(26.4)	22(18.2)	67(55.4)	0.08(0.175)
Patients present with better oral hygiene when treated with	52(43)	3(2.5)	66(54.5)	0.40(<0.0001)
Assistants prefer working with	36(29.8)	21(17.4)	64(52.9)	0.12(0.046)
Adjustment appointments are shorter with	58(47.9)	18(14.9)	45(37.2)	0.33(<0.0001)
For a crowded dentition, I would be less likely to extract teeth using	54(44.6)	8(6.6)	59(48.8)	0.38(<0.0001)
Initially, treatment progresses faster with	66(54.5)	7(5.8)	48(39.7)	0.49(<0.0001)
There are fewer emergency visits with	26(21.5)	8(6.6)	87(71.9)	0.15(0.002)
Long-term stability with less relapse potential is better achieved with	4(3.3)	18(14.9)	99(81.8)	-0.12(0.003)
Which bracket system is most cost effective?	20(16.5)	71(58.7)	30(24.8)	-0.42(<0.0001)
Indicate which type of brackets you prefer to use for space closure	17(14)	73(60.3)	31(25.6)	-0.46(<0.0001)
Indicate which type of brackets you prefer to use in finishing and detailing	10(8.3)	90(74.4)	21(17.4)	-0.66(<0.0001)

When orthodontists were asked about stability, 99% of the respondents reported no difference which agreed with Prettyman et al.'s findings (93% of their sample reported no difference in stability between the two types). A long follow-up retrospective study compared stability between patients treated with CB SLB and reported that there was no statistically significant difference in long-term stability between the two systems (Yu et al., 2014). Most of our sample reported that they prefer CB for finishing and detailing 74.4% (P<0001), which agreed with previous study where 64% (P<0.0001) of their sample preferred it (Prettyman et al., 2012).Furthermore, finishing and detailing were disadvantages reported from SLB as a result of greater clearance between the bracket a lot and the archwire (Harradine and Birnie, 1996).

SLB are expensive compared to CB. Many orthodontists are looking to increase clinical efficiency to justify the increased cost (Marshall et al., 2010). The majority of our orthodontists (73%) reported that CB was more cost-effective compared to SLB in accordance with Prettyman et al. and Rathi et. al. where their samples reported that CB were more cost effective (70% and 68% respectively) (Harradine and Birnie, 1996; Prettyman et al., 2012).

There are different brands of SLB; there are also active and passive SLB, each has different characteristics that affect clinical performance which was evaluated in this study. This should be kept in mind when interpreting study results, as questions on SLB did not specify name or type. The study evaluated the perception of orthodontists regarding SLB and CB; therefore, the orthodontists' answers might be biased by the bracket system they use in.

CONCLUSION

Participating orthodontists perceived a clinical difference between SLB and CB. Orthodontists preferred SLB in 7 out of the 12 treatment factors that were evaluated. SLB were preferred mostly for initial treatment progress, shorter adjustment appointments, oral hygiene, and less required extraction. CB were preferred mostly for space closure, finishing, and cost-effectiveness according to the respondents.

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Effect of Glycated Hemoglobin Levels on Intraocular Pressure in Patients with Diabetes Mellitus in Saudi Population

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ABSTRACT

Aim of the study was to investigate the effect of chronic hyperglycemia as determined by high glycated hemoglobin (HbA1c) on intraocular pressure (IOP) in patients with diabetes and to recognize the diabetic patients at high risk of developing glaucoma in a tertiary care hospital in western region of Saudi Arabia.This was a retrospective chart review performed at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. Hospital records of diabetic patients in the department of ophthalmology from August 2015 to June 2020 were collected. Patients diagnosed with glaucoma, using intraocular pressure-lowering medications, or using topical or oral steroids were excluded from the study.Overall, 159 participants were enrolled in the study. A significant association between high HbA1c levels and IOP values was observed. Individuals with HbA1c below 6.5, between 9.6 to 10.5, and over 12.6 had a mean IOP of 15.2 ± 2.87, 16.6 ± 5.12, and 19.5 ± 1.88, respectively (p= 0.031). Longer diabetes duration was associated with a higher IOP (p=0.028). Another finding illustrated that female participants had significantly higher IOP compared to males (16.94 ± 3.25 mm Hg, 15.15 ± 3.31 mm Hg, p=0.001, respectively). A significant positive association between high HbA1c levels and IOP values was found, which indicates that diabetes and elevated HbA1c are significant contributing factors for elevated IOP. There was a statistically significant higher IOP in females in which further research is needed with prospective and extensive data collection. Accordingly, a regular diabetic eye examination to monitor intraocular pressure is recommended specially to those with uncontrolled diabetes and high HgA1c to reduce ocular morbidity due to glaucoma.

KEY WORDS: DIABETES; INTRAOCULAR PRESSURE; GLAUCOMA, GLYCATED HEMOGLOBIN.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease associated with chronic hyperglycemia (Mayer-Davis et al .2018). It is a distressing epidemic and is considered one of the leading causes of death worldwide (Glovaci et al. 2019). Globally, the prevalence of DM is estimated to be 9.3% (463 million people) in 2019, increasing to 10.2% (578 million)and 10.9% (700 million) by 2030 and 2045, respectively, with more than 29% incidence in Saudi Arabia alone from 1990 -2015 (Saeedi et al. 2019) (Alotaibi et al. 2017). DM is diagnosed according to plasma glucose criteria in the form of fasting plasma glucose (FPG) levels, 2-h plasma postprandial glucose (2-h PPG) levels, or the glycosylated hemoglobin (HbA1c) criteria reflecting the average plasma glucose concentration over the previous 8–12 weeks (Care 2019) (Nguyen et al.2019). The International Expert Committee recommends HbA1c as a reliable tool for diagnosing type 1 and type 2 DM with a cutoff point of ≥6.5% (Nathan et al. 2009). Al Salamah et al.(2020) have reported that 35% of Saudis aged 55 or more had type 2 diabetes.

HbA1c testing has multiple advantages over plasma glucose measurement, such as pre-analytical stability and less day-to-day variation due to stress or illness (Nathan et al.2009). Therefore, HbA1c is the gold standard for diabetes control. Besides reflecting the glycemic adjustment, Hba1c is used as a predictor 1to assess secondary microvascular complications, including retinopathy, neuropathy, and nephropathy in cases of insufficient glycemic control (Hasselacher et al. 2014). Diabetes contributes to the risk of developing several types of glaucoma, most commonly, primary open-angle glaucoma (POAG) and neovascular glaucoma (NVG) (Resnikoff et al.2004 Barac et al. 2015, Bahera et al .2020).

POAG is a multifactorial disease that is caused by retinal ischemia, remodeling of the optic nerve head, and altered trabecular meshwork function(Feki et al. 2019) (Faralli et al. 2019) . Diabetic patients are susceptible to retinal ischemia, which is believed to be the main cause of neovascular glaucoma by stimulating the release of vascular endothelial growth factor-A (VEGF-A), leading to vasodilatation and increasing blood flow, which initiates new blood vessel formation leading to NVG (Hayreh 2007, Yang et al .2018). Glaucoma is defined as a group of ocular disorders that are characterized by progressive optic neuropathy and associated visual field loss (Bertaud et al. 2019). Although treatable, it is the most common irreversible blinding disease worldwide (Quigley et al. 2006). Therefore, early detection is required for a good prognosis. Normal intraocular pressure (IOP) is 10-21 mm Hg, which is preserved by a balance between the aqueous humor production and drainage. Any imbalance leads to elevated IOP (Khaw and Elkington 2004), causing both vascular and mechanical stresses (Song et al. 2016) .Therefore, it is an important risk factor for glaucoma deterioration and progression, and currently, is the only modifiable factor (Asal et al. 2020).

A recent meta-analysis evaluated 47 studies from 16 different countries and found that patients with diabetes had been associated with an average of 0.18 mmHg increase in the IOP (Zhao et al. 2014) Furthermore, other studies found that patients with increased levels of HbA1c had substantially higher IOP levels compared to the patients with lower levels of HbA1c (Hymowitz et al. 2016, Perez-Rico et al. 2015, Takahashi et al. 2020).

A study conducted in Riyadh, Saudi Arabia, found that diabetic patients had higher IOP compared to non-diabetic subjects. HbA1c was used as a criterion for diagnosing diabetes; however, the relationship between HbA1c value and IOP has not been studied (Briggs et al. 2016). To the best of our knowledge, there have been no reports evaluating the relationship between HbA1C and IOP among the Saudi population. Therefore, we aimed to investigate the effect of chronic hyperglycemia as determined by HbA1c on IOP in patients with diabetes and identify diabetic patients at risk of developing glaucoma in Saudi Arabia from 2015 to 2020.

MATERIAL AND METHODS

Study design and setting: This retrospective chart review study was conducted at King Abdul-Aziz University Hospital (KAUH), a tertiary center in Jeddah, Saudi Arabia. Medical records from glaucoma and retina clinics in the Department of Ophthalmology between August 2015 and June 2020 were collected.

Sample criteria and diagnostic instrument: Patients aged 15-90 years diagnosed with type 1 or type 2 DM were included Patients diagnosed with glaucoma, using IOP-lowering medications, or using topical or oral steroids were excluded from the study. Of the 383 diabetic patients treated at the department of ophthalmology between 2015 to 2020, 224 subjects were excluded: 73 diagnosed with glaucoma, 138 with previous history of laser or intraocular surgery, and 13 on IOP-lowering medications or topical steroids. Thus, 159 subjects met the inclusion criteria and were enrolled in the study.Data obtained from medical records included demographic data such as age, sex, and nationality. Additionally, type and duration of diabetes, HbA1c levels, IOP in the right and left eye (IOP-OD, IOP-OS, respectively), and body mass index (BMI), which was calculated as weight in kilograms divided by height in meters squared were also collected.

The patient's IOP was measured using a Goldmann applanation tonometer. The mean IOP was calculated for each patient as the sum of the pressure of both eyes divided by two, using an excel equation. Glycemic control measurement (HgA1c), was obtained within one year before or after IOP measurement. Patients were categorized according to their glycemic control in three categories: good glycemic control (HbA1c <7%), moderate glycemic control (HbA1c 7-9%), and poor glycemic control (HbA1c >9%) (23). Diabetes duration was defined as the period from the first diagnosis to the day of IOP measurement.

Analysis:Data were registered using an online Google form, and was then imported to Microsoft Excel 2020 for data entry. Statistical analysis was performed using the Statistical Package for the Social Sciences IBM© SPSS© version 21 (IBM© Corp., Armonk, NY, USA). Descriptive statistics (mean and standard deviation) were calculated for normally distributed variables including IOP, HbA1c, BMI, age, and diabetes duration. Frequencies and percentages were calculated for sex, nationality, diabetes type, and population categories. We used the Shapiro-Wilk test to check for normality. An independent-samples t-test was used to compare the IOP in both sexes and both types of diabetes. For multiple comparisons with the IOP, one-way analysis of variance (ANOVA) was performed. All P-value < 0.05 were considered to be statistically significant. Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Research ethics: This research was approved by the Biomedical Ethical Committee at KAUH (ref: 653-19).

RESULTS AND DISCUSSION

There were 73 (45.9%) men and 86 (54.1%) women. The mean age was 58 ± 16 years, with the majority of patients being 50 to 69 years old (49.1%). The majority of patients, 82 (51.6%), were type 2 diabetic patients. The characteristics of the study population are shown in Table 1.

Table 1. mean and standard deviation of the study population characteristics

Characteristics	N(%)	Mean ± SD	IOP (mm hg)	
			Mean ± SD	p-value
Gender				
Male	73 (45.9%)		15.15 ± 3.31	0.001
Female	86 (54.1%)		16.94 ± 3.25	
Diabetes type				
Type 1	77 (48.4%)		16.38 ± 3.14	0.347
Type 2	82 (51.6%)		15.87 ± 3.60	
BMI classifications	4 (2.5%)	17.53 ± 0.64		0.77
Underweight	34 (21.7%)	22.49 ± 1.57		
Normal weight	38 (24.2%)	27.51 ± 1.43		
Pre-obesity	81 (51.6%)	35.55 ± 4.76		
Obesity				
Age classifications				
<29 y	15 (9.4%)	23.47 ± 3.96		0.42
30 – 49 y	23 (14.5%)	42.04 ± 5.42		
50 – 69 y	78 (49.1%)	60.90 ± 5.08		
70 – 84 y	43 (27.0%)	75.91 ± 4.84		

Female participants had statistically higher IOP compared to the male participants (16.94 ± 3.25 mm Hg, 15.15 ± 3.31 mm Hg, respectively, p=0.001). We observed no significant difference between Type 1 as well as Type 2 DM and IOP (16.38 ± 3.14, 15.87 ± 3.60, respectively), p=0.347. When we classified diabetic patients according to their glycemic control, 72 participants (46.5%), almost half of the sample, had moderate glycemic control. The mean HbA1c for good, moderate, and poor controls was 6. ± 1.01, 8.05 ± 0.58, and 10.80 ± 1.40. respectively. The mean IOP was elevated in the poor glycemic control patients (16.78 ± 3.65 mmHg) compared to those with good glycemic control (15.65 ± 3.08 mm Hg). The difference was not statistically significant (p=0.263). Table 2 shows a comparison between the three groups.

There was a statistically significant difference between the HbA1c range groups and IOP, as determined by one-way ANOVA (F [7,149] = 2.276, p = 0.031). Subjects with HbA1c above 12.6 displayed higher IOPs (19.58 ± 1.88 mm Hg), compared to the subjects with HbA1c between 6.5 to 7.5 (15.52 ± 3.89 mm Hg), and between 7.6 to 8.5 (16.029 ± 2.81 mm Hg), with a mean difference of (4.05 mm Hg), (p=0.006) and (3.55 mm Hg), (p=0.017), respectively (Figure 1). One-way ANOVA revealed that with longer duration of diabetes, the IOP significantly increased as well (F (3,143) = 3.117, p = 0.028). Thus, participants with a diabetes duration of less than five years had a mean IOP of 14.75 ± 2.99 mm Hg, while participants with a diabetes duration ranging from 11 to 20 years had a mean IOP of 16.93 ± 3.79 mm Hg, with a mean difference of (2.21 mm Hg), (p=0.006). Table 3 presents the mean IOP values according to the duration of DM.

In this study, we found that individuals with higher levels of HbA1c exhibited significantly higher IOP levels compared to individuals with lower HbA1c levels. This

result is consistent with those of previously published studies, (Kang et al. 2019,Takahashi et al. 2020). Hymowitz et al.2016 found an association between poor glycemic control, as evidenced by higher HbA1c and elevated IOP. Baisakhiya et al. 2017 observed an association between higher HbA1c and raised IOP, reporting that poor glycemic control is a risk factor for glaucoma in diabetic patients.

The underlying pathogenesis that explains how DM promotes increased IOP remains unclear. Diabetes is a known cause of microvascular damage and can disturb blood flow at the level of the optic nerve head and retina, which stimulates the invasion of the iris surface and iridocorneal angle of the anterior chamber by a fibrovascular membrane. This fibrovascular membrane initially resists the aqueous outflow, resulting in open-angle glaucoma, and later obstructs the angle and produces secondary angle-closure glaucoma, (Hayreh 2007,Salzy et al.2009, Grzybowski et al. 2020). In addition, higher glucose levels in the aqueous humor of diabetic patients have been observed to upregulate and promote the accumulation of extracellular matrix proteins, particularly fibronectin, thereby blocking aqueous drainage, leading to an increase in IOP. Chronically raised IOP levels sequentially lead to optic nerve head damage, stemming from progressive mechanical compression (Faralli et al .2019).

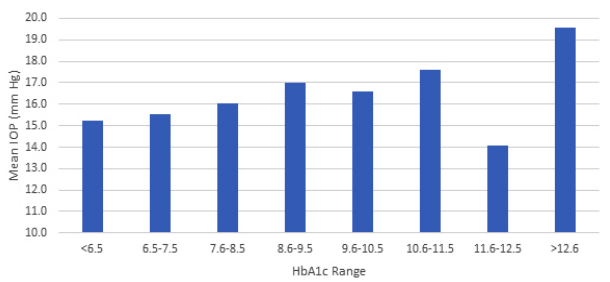
Table 2. mean and standard deviation of HgA1c and IOP in glycemic control groups

	good glycemic control: N (%)38 (24.5%) Mean ± SD	moderate glycemic control: N (%)72 (46.5%) Mean ± SD	poor glycemic control: N (%) 45 (29%) Mean ± SD	p-value
HbA1c	6.006 ± 1.014	8.052 ± 0.581	10.809 ± 1.405	0.001
IOP	15.65 ± 3.08	15.91 ± 3.39	16.78 ± 3.65	0.263

Table 3. Mean and standard deviation of IOP according to duration of DM

Diabetes duration	N(%)	Mean ±SD	Mean IOP (mmHg) ±SD	P value
<5 y	26 (17.7%)	2.04 ± 1.34	14.75 ± 2.99	0.028
5 – 10 y	33 (22.4%)	8.91 ± 1.73	15.74 ± 2.45	
11 - 20 y	57 (38.8%)	16.25 ± 2.708	16.93 ± 3.79	
21 – 33 y	31 (21.1%)	24.58 ± 3.09	16.82 ± 3.46	

Figure 1: Comparison between HgA1c range and IOP



Oshitari et al. (2007) found a significant association between IOP and glycemic control categories, which is inconsistent with our result, as we could not find an association between glycemic control categories and IOP levels. This is probably due to the use of different HbA1c classifications. Moreover, some researchers have found an alteration in the IOP values following transient blood glucose fluctuations in cases of hypoglycemia or postprandial hyperglycemia that cannot be measured by HbA1c (Rihan et al.2020). Additionally, comorbidities that falsely lower HbA1c levels on test results by shortening the erythrocyte survival rate could potentially affect the outcome of the study (Report 2011, Ang et al. 2014).

Our study showed that people with a longer duration of diabetes had higher IOP values compared to patients with a shorter duration. In contrast, a previous study conducted in Riyadh, Saudi Arabia, stated that the duration of diabetes did not vary significantly with IOP levels (Briggs et al. 2016). Recall bias of diabetes duration plays a major role in the inconsistency between the two results. Further, a meta-analysis study reported that each year since diabetes diagnosis increases the risk of glaucoma by 5% (95% CI, 1%–9%), which may be due to the cumulative neuronal damage that progresses with time, (Zhao et al. 2015, Grzybowski et al .2020).

Moreover, we found that female participants had a higher IOP than males, a difference which was statistically significantly. Moreover, Kang et al.2019 reported a significant relationship between IOP and HgA1c in female without diabetes. In contrast, Hymowitz et al.2016 reported no significant difference between two sexes regarding IOP levels. However, variability in the results

could stem from differences in the male-to-female ratio between the two sample populations. The sex-related differences in IOP measurement remain incompletely understood. Previous studies reported that anatomical and structural eye differences between the two sexes made the comparison inaccurate, which explains part of this discrepancy (Patel 2018, Chua et al. 2019).

Limitations: This retrospective record review study encountered a few inherent limitations. First, it was not possible to provide precise HbA1C levels and random blood glucose at the exact time point during which the IOP was measured. In addition, open-angle glaucoma has no symptoms initially prior to peripheral vision loss; therefore, many diabetic patients had already presented with glaucoma due to delayed periodic eye screening.

Recommendation Planning an educational campaign empowering diabetic people to perform periodic eye screening for monitoring IOP to ensure that glaucoma can be diagnosed in the early stage and effectively treated. Further, future studies to assess the confounding factors that may influence the association between HbA1C and IOP, such as central corneal thickness and lens status are warranted.

CONCLUSION

The current study found a significant association between high HbA1c levels and IOP values, which indicates that diabetes and elevated HbA1c are significant contributing factors for elevated IOP. Therefore, a well-established diabetic screening program includes IOP measurement should be applied routinely. Accordingly, diabetics with uncontrolled blood sugar and high HgA1c are recommended to undergo more frequent eye examination to monitor IOP to reduce ocular morbidity due to glaucoma.

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Relation Between Physical Activities and Dietary Habits in School Children of Riyadh, Saudi Arabia

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ABSTRACT

Physical activity and dietary habits during childhood play a major role in affecting physical, mental, and cognitive factors in adulthood. Physical inactivity and unhealthy diets may lead to health issues in the future. This study aimed to investigate the relation between dietary habits and physical activity among school children in Saudi Arabia. This cross-sectional study was performed in the capital city, Riyadh of Saudi Arabia and students included in the study were between 5 and 13 years of age who were recruited through an online questionnaire. A total of 122 Saudi students, including 46.8% boys and 53.2% girls, were selected from various schools in Riyadh city. Data on the dietary habits and physical activities of the school children were collected via a questionnaire. The results of the study confirm that 77% of children had a habit of eating fruits and vegetables. Above 60% of children had a habit of drinking soft drinks, and 84.4% of children showed an interest in eating fast food. Approximately 97% of children were addicted to eating snacks and the majority of students appeared to eat 1 h before going to bed in addition to having a habit of eating late dinners. The students were also actively involved in physical activities with 94.3% of the participants undertaking 1–6 h of physical exercise per week, including running and walking. In conclusion, the current study confirms a significantly high correlation of physical activity on consumption of excess fruits and vegetables as part of a dietary pattern and also on consuming high levels of fast foods and soft drinks. One of the major concerns is connected because of late dinner pattern.

KEY WORDS: DIETARY HABITS, PHYSICAL ACTIVITY, FRUITS, VEGETABLES, SCHOOL CHILDREN.

INTRODUCTION

Physical activity is defined as the skeletal muscular movement of a body which results in energy expenditure. The extensive benefits of participating in physical activities for children include positive physical self-concept, better academic outcomes, boosted global self-esteem, and improved physical and mental health, (Sneck et al., 2019). Physically active students have high energy intakes which balance energy expenditure through a healthy, balanced diet which further provides energy to manage stress (Caldwell et al., 2019). Low intake of dietary fat and high consumption of fruits and vegetables along with increased physical activities can lead to a healthy life without adverse effects during adolescence (Popkin et al., 2012). School children with low levels of physical activity are more likely to develop serious health issues in the long-term such as heart disease and weak bones (PeopleandServices, 2020).

A lack of physical activity can also lead to obesity, which is a commonly known risk-factor associated with low levels of physical exercise (Sun et al., 2020). A strong link was identified between health and education, which is associated with schools promoting physical activity in their students (Tercedor et al., 2017). Physical education

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plays a major role in an advantageous position for endorsing leisure benefits of physical activities (Polet et al., 2019). The World Health Organization (WHO) has stated that globally, nearly 1.9 million deaths are accredited to physical activity, and they recommend school children should participate in 60 min per day ophysical activity (Dobbins et al., 2013). Schools play an important role in creating a safe and caring environment that sustains healthy practices and also provides opportunities for students to learn and practice healthy eating habits and encourages regular physical activity Morbidity and Mortality Weekly Report (2011). Physical inactivity combined with sedentary factors and poor diet can lead to weight gain in children (Pozuelo-Carrascosa et al., 2018). The WHO confirms that physical inactivity in the fourth leading cause of global mortality (Alahmed et al., 2018).

Nutritional dietary habits in children are major developmental factors for improving physical, mental, and cognitive levels during adolescence (Kristo et al., 2020). A healthy diet is also used to prevent malnutrition and non-communicable diseases (NCDs). The consumption of processed foods can often modify individual lifestyles, including dietary patterns (Breda et al., 2019). The habit of consuming a balanced and healthy diet starts during childhood can prevent the development of NCDs and chronic diseases in adolescence and adulthood (Tambalis et al., 2019). Acidogenic foods with rich diets include animal meat, fish, cheese, rice, and cereals, and low alkaline foods such as fruits and vegetables support endogenous acid production. Conversely, poor diets and abnormal lipid profile are associated with the development of obesity and cardiovascular diseases (Aslani et al., 2020).

Healthy nutritional diets in small-aged school children safeguards enhanced health status during adulthood (Soheilipour et al., 2019). Breakfast is considered as the first meal of the day and should be considered a healthful diet as well as lifestyle which further can absolutely impact children's health, specifically, when involving high nutritional fiber, fruits, whole-grain, vegetables and regular products (Yu et al., 2019). But in a recent trend, some nuclear families have modified their lifestyle, including dietary habits. Fast food restaurants are one of the environmental factors that interact with particular characteristics to effect individual weight status. For example, fast food restaurants located near schools can influence body mass index (BMI) outcomes. Junk fast foods are high calorie foods that cause weight gain, resulting in obesity (Asirvatham et al., 2019, Jia et al., 2019).

The WHO estimates that 57% of children in Saudi Arabia are currently physically inactive (Alahmed et al., 2018). Limited studies have been documented about physical activity and other related factors in Saudi school children (Al-Hussaini et al., 2019); there are limited studies in adults, children, and expatriate populations from different regions of Saudi Arabia about a combination of physical activity factors (Al-Hazzaa et al., 2011, Darwish et al., 2014, Alzeidan et al., 2017). To the best of our knowledge, there are currently few studies on school children in the age range of 6–13 years with the combination of physical activity and dietary habits from the Kingdom of Saudi Arabia. Therefore, the current study aimed to determine the association between physical activity and dietary habits in Saudi school children.

MATERIAL AND METHODS

Design of the study: This cross-sectional study was conducted at the female campus of the Department of Community Health Sciences in the College of Applied Medical Sciences at King Saud University in the capital city of Saudi Arabia. A total of 122 children (age range, 5–13 y) were recruited for this study based on a multistage stratified sampling technique. The study participants confirmed their informed written consent (via parent or guardian) before participating in this study. This study was designed as a cross-sectional analytical pilot study performed in Saudi school children to record their BMI, dietary habits, and physical activities.

This study was performed in Riyadh city schools of the north, west, south, and eastern regions from international, non-profit, and national private schools of each region. The inclusion criteria were as follows: children from Riyadh prominence region with an age range of 5–13 years. The exclusion criteria: child with chronic diseases, child above or below the age range. A total of 122 samples were included as the final subset (46.8% boys and 53.2% girls). However, 22 students were excluded owing to unsigned consent forms.

Dietary habits: In this study, dietary habits regarding consumption of fruits, vegetables, junk food, soft drinks and snacks were recorded on a weekly basis.

Physical activities: The student's physical activities were recorded, including going to the gym, running, walking, involvement in physical activity classes conducted within school premises, and irregular physical activities.

Anthropometric and other measurements: Obesity was measured by BMI through the combination of weight in kg and height in cm. BMI classifications are based on WHO BMI for ages 5–19 years. Based on the BMI values, children were categorized as underweight, normal-weight, overweight, or obese (Khan et al., 2019).

Statistical analysis: Descriptive statistics were provided as means and standard deviations. Data was analyzed to examine the risk of obesity in Saudi children based on dietary habits and physical activities using SPSS version 15.0. Results were presented as percentages (%).

RESULTS AND DISCUSSION

In this cross-sectional study, a total of 122 students were recruited based on an online questionnaire form. The basic and anthropometric details of school children are presented in Table 1. Both girls and boys aged between

5 and 13 years were included in this study. The mean age of the total students was 9.41 ± 2.61. The results indicated that 54.9% of girls were more actively involved in the physical activity when compared to 45.1% of boys. The mean height and weight of both girls and boys was 125.52±24.3 and 35.1±17.9. The mean total BMI was found to be 22.6 ± 11.56 kg/m2. In this study, 12.3% of children were affected with different diseases such as asthma, eczema, allergy, cramps, herpes, epilepsy, epistasis, anemia, and hyperactivity.

Table 1. Basics characteristics of school children

Basic characteristics	Frequencies	Percentages
Age (Years)	9.41 ± 2.61	NA
Sex (Male: Female)	55: 67	(45.1%): (54.9%)
Weight (Kg)	35.1 ± 17.97	NA
Height (cm)	125.52 ± 24.3	NA
BMI (kg/m2)	22.6 ± 11.56	NA
Child affected with	(12.3%)	
specific disease	15	
Educated mothers	122	100%
Educated fathers	122	100%
Filled questionnaire	122	100%
Mother	105	86.1%
Father	03	2.5%
Sister	08	6.6%
Brother	02	1.6%
Grand mother	02	1.6%
Grand father	00	0%
Aunt	01	0.8%
Uncle	01	0.8%

Table 2. Categorization of children's body mass index (BMI)

Types of BMI	Frequencies	Percentages
Underweight	55	45.1%
Normal	29	23.8%
Overweight	23	18.8%
Obesity	15	12.3%
Morbid-Obesity	00	0%

The literacy rate for the children's parents was found to be 100%. In this study, a maximum of 86.1% of questionnaires was filled by the mother and remaining 13.9% of questionnaires was documented by father, brother, sister, grandmother, aunt, or uncle. Table 2 shows the BMI categories. Almost 45% students were in the underweight category, 23.8% in the normal-weight category, 18.8% in the overweight category, and 12.3% were obese. In this study, there was no student in the morbid-obesity category. The complete details collected regarding food habits in the children are summarized in Table 3. In this study, 77% of children had the habit of eating fruits such as dates, oranges, apples, bananas, and mangoes. Almost 76.2% of children reported eating healthy vegetables, i.e., cucumber, carrot, tomato, lettuce, and other greenery on a daily basis. More than 60% of children had the habit of drinking soft drinks, and 84.4% of children showed an interest in eating fast foods. Nearly 97% children were addicted toward snacks such as sweets, chips, cakes, crepe cookies, doughnuts, chocolates, and sweetened carbonated drinks. All children had the habit of consuming foods at different times before going to bed. A total of 23.8% of children indicated consuming dinner about less than an hour before bedtime, 41% of children indicated eating dinner an hour before going to bed, and approximately 30.3% of children indicated eating dinner 2–3 h before going to bed. Only 3.3% of children indicated having dinner 4 h before going to bed and finally 1.6% of children indicated having dinner more than 4 h before going to bed (Table 3).

Table 3. List of children's dietary habits

Dietary habits	Frequencies	Percentages
Habit of eating fruits	94	77%
Habit of eating vegetables	93	76.2%
Habit of drinking soft drinks	74	60.7%
Habit of eating fast foods	103	84.4%
Habit of eating snacks	118	96.7%
Dinner time (<1–4+hrs)	122	100%

Table 4. List of physical activities within school children

Activities	Frequencies	Percentages
Physical activity	115	94.3%
Physical effort	105	86.1%
Gym	14	11.5%
Running	115	94.3%
Walking	115	94.3%
Physical activity classes	88	72.1%
Irregular physical activity	34	27.9%

The list of physical activities that the children participated in is detailed in Table 4. In this study, 94.3% students were found to be actively involved in physical exercises, including running and walking, on a weekly basis. On regular basis, 6.5% students performed physical activities for a minimum of 1 h, 3.3% for a couple of hours, and 0.8% for 3 h regularly. Only 2.4% of children performed physical activities within their school premises. There were approximately 4.1% children who performed physical activity for 30 min. Approximately 33.6% students performed physical activity on a weekly basis for 1 h; 10.6% for 2 h; 11.5% for 3 h; 4.9% for 4 h; 4.1% for 5 h; 3.3% for 6 and 7 h; 1.6% for 10 h; and 0.8% for 8, 9, 12, 14, or 17 h. In total, 86.1% students made physical efforts and 13.9% did not. However, 38.6% made the effort 1–3 times/week, 13.1% did 4–6 times/

weeks, and 34.4% did on a daily basis. However, 13.9% students were motivated by family members.

Additionally, 11.5% students participated in the gym and 88.5% were not involved owing to the restricted age limit. Approximately, 72.1% students participated in physical activity classes conducted by their schools and among these, 36.1% and 18% participated for 1-2 and 3-4 times on a weekly basis, respectively and 18% actively participated on a regular basis. There were approximately 19.7% children who were unable to participate owing to a lack of physical activity sessions in a limited number of schools and only 8.2% students did not participate due to the inactive.

The purpose of designing this study involving school children between the age of 5 and 13 years is that both male and female students have maximum prospects for contributing to numerous types of physical activities in their routine life. This study aimed to investigate the relation between dietary habits and physical activity among Saudi school children. The results confirmed that >76% of Saudi children prefer to eat vegetables and fruits on a weekly basis, and almost 85% students prefer fast food and snacks. Additionally, the results highlighted that 94.3% students were actively involved in physical activity for 1–6 h per week.

In this study, 31% children were considered to be overweight and/or obese, and approximately 31% of the remaining children were found to be normal with the underweight criteria. Limited studies have been performed within this population of school children in Saudi with the combination of factors of physical activity and sedentary lifestyle related to other factors such as BMI, weight gain as overweight and obesity (Darwish et al., 2014, Al-Husaini et al., 2019, ElkhodaryandFarsi, 2017, Al-Nuaim et al., 2012, Alqahtani et al., 2015). A previous study with Saudi children and adolescents has confirmed with limited physical activity (Al-Hazzaa, 2002) and the same author previously conducted a similar study within the Saudi population and documented a 43.3–99.5% prevalence of physical activity (Al Hazzaa, 2004).

A previous study from Saudi Arabia has also concluded that 11%, 42.7%, and 46.3% of children spend 1–<2, 2–4, and >5 h in playing activities, (Darwish et al., 2014), whereas in our study, 33.6% students performed physical activity on a weekly basis for 1 h; 10.6% for 2 h; 11.5% for 3 h, 4.9% for 4 h, and 4.1% for 5 h. The WHO guidelines strongly recommend sustaining physical activity for a minimum of an hour per day in children to prevent certain diseases, (OjaandTitze, 2011). Physical activity is positively associated with lowering the risk of cardiovascular diseases and mortality, (Lind et al., 2017).

Physical inactivity is associated with the prevalence of weight gain, i.e., overweight or obesity in the children and in our study, only 18% of children were involved in sports activity for an hour and above in a day, which is less than a quarter of the sample, whereas the percentage of obesity and excess weight in our study did not exceed 30%. The 36.6% of students were addicted toward an electronic gadget or watching television for a couple of hours and 33.1% of these spent minimums of 3-4 h on daily basis. Al-Hazzaa et al (2006) performed a study with school children of Saudi Arabia and concluded that an increase in physical activity lowers the chances of developing obesity. Biddle et al (2017) systematically reviewed involving 29 original global studies and concluded that there is a limited association between sedentary behavior and adiposity in both children and adolescents. Zurita-Ortega et al (2017) conducted a study with children in Chile and confirmed that self-esteem was positively associated with physical activity engagement.

A similar follow-up study should be implemented in Saudi community to evaluate the social effects on physical activity. Based on the context of nutritional patterns, this study has documented the expected results on the behavior and choices of children who were enrolled in this study. The results of this study highlight that >75% students had a habit of regular consuming fruits and vegetables and 60%–84% students were addicted to soft drinks and fast foods. The data of the present study are also positively associated with those of previous studies performed by Luszczki et al (2019) and Kristo et al (2020).

The results of our study are similar to those of Nakahori et al., (2016). Numerous studies have been performed in different populations comprising school-going children aged 6–13 years and documented unhealthy eating habits with less physical activity which was shown to be associated with human diseases, (Vilchis-Gil et al., 2015, Lopez-Sobaler et al., 2003). However, based on the literature, several inconsistencies were recorded on the results and dietary effects in children. Extensive research with appropriate sample population is required to shed further insight on the matter.

One of the important issues raised in this study is the time at which dinner is consumed in relation to when children go to bed. Results indicated that 23.8% of children consumed dinner 30 min before bedtime, 41% did so 1 h before bedtime, 30.3% did so 2–3 h before bedtime, and 3.3% consumed dinner 4 h before bedtime, and 1.6% of children did so >4 h before bedtime. The majority of students appeared to eat 1 h before going to bed for sleeping. Okada et al (Okada et al., 2019) conducted a study on the habit of eating late dinners in 19,687 Japanese women between the ages of 40 and 74 years and confirmed that 11% of them had late dinners on a daily basis, 22% of women consumed snacks during bedtime, and 8% of women skipped breakfast and these lead to weight gain. Bo et al (Bo et al., 2014) confirmed that the consumption of excess calorie intake on a daily basis before bedtime may lead to an increased risk of obesity, metabolic syndrome, and/or non-alcoholic fatty liver disease.

CONCLUSION

The strength of the present study is to involve complete Saudi ethnicity of school children. The record of 7 days/ week helped us document the dietary habits and physical activities (Table 3 and 4) of the children in the study; finally, we have documented the BMI of the student. One of the limitations of this study is the low sample size with limited information. In conclusion, the current study confirms a high prevalence percentage of physical activity and consumption of excess fruits and vegetables along with the habits of consuming soft drinks and fast and junk food, including chips, which may belong to nuclear families. There is a need for future studies with large sample sizes, and it is recommended that restrictions be put on children consuming junk food, late night meal and soft drinks.

Conflict of Interest: None

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Evaluating the Influence of Physical Education Programs Based on A Game Approach to the Actual Motor Capacity of Elementary School Children

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ABSTRACT

The present study was carried out to test the effectiveness of the program on the development of the basic motor skills by taking into account the degree of movement in overtime activities of sampled participants and their gender. The study included 74 children (36 males and 38 females; average age: 9 years). According to these data, children were grouped into two moving levels (i.e., High and Low) and, for each group by sex, a t-sample test was performed to assess the level of actual motor capacity development before and after the project. Males who were brought into the low-level movement group had a significant improvement in the location and the coarse engine development negotiation score (which is most likely and likely to have a positive effect). A contradictory effect is estimated for women: only girls in high-level movements show significant and likely positive effects of treatment in their motor scores. These results highlight the effect of analyzing the physical education program, although the relative positive results on the selected participants are not enough to overcome the problems of child malnutrition that the document always emphasizes.

KEY WORDS: CHECK FOR DEVELOPMENT OF GROSS ENGINE; TEACHING METHODS; EXTRACURRICULAR ACTIVITIES; TEACHING-LEARNING PROCESS.

INTRODUCTION

It is well known that the *fundamental motor* skills (FMS) is related with the physical activity and the sport practice. Particularly, the importance of motor development and its influence on the physical activity were regarded as the focus point of the research. In this respect, some authors hypothesized that a greater commitment in physical activity is linked with a good level of FMS (Stodden et al., 2008) and that a child skilled in physical activities will be more active people as well (Clark, 2005). The importance of FMS is also supported by the large amount of studies that investigate several aspects related to the teaching-learning process, such as the usefulness of several and various assessment methods (Sgrò et al., 2016, Logan et al., 2015; Francesco et al., 2019).

Several of reasons have been pointed out as the cause of this problem: the time of playing video games, low availability of physical activity and sport fields, and the low level of physical and sporting education in school time. In contrast, the school is widely recognized as an important environment to support skills, knowledge and behavior towards physical activity and sports throughout

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life. In this regard, *physical education* (PE) lessons require high quality teaching methods, more lessons per week and a strong relationship with extra-school physical activity and sports clubs for providing additional opportunities.

In Vietnam, as in other Asian countries, primary education lessons on the primary level are not given to well-trained, irregular teachers and the effects of those lessons on delivery. The development of the full FMS level is significantly limited. Recently, to overcome the above problems, a physical education project has been supported and implemented by the Vietnam National Ministry of Education, University and Research and the Olympics. Therefore, by considering similar initiatives that can be implemented in the following years as well as in other Asian countries, the purpose of this study is to examine the effectiveness of the above project for with the actual motor capacity of students who participate in related physical education and sports lessons by taking into account the level of movement in overtime activities of sampled participants and the gender.

MATERIAL AND METHODS

The participants of this study were sampled from a primary school of a city located in the South of Vietnam. The participants were 74: male: 36; female:38; mean age:9 years old; mean height: 1.34 m; mean weight: 35.02 Kg. The participants were involved in a physical education curriculum that will be described in the following sections of this manuscript. The project is managed for 5 months and the levels of actual motor competences were verified pre- and post-project' teaching activities. Assessment procedures were performed at the school gym, where almost four skilled operators met the participants. Overall, the evaluation time is six days. For each day, to ensure valid and reliable assessment procedures, participants are divided into the smallest group (i.e., five children per group). Anthropometric measures are performed from the same operator for all participants using a wall mounted meter for height and electronic weighing scales. These measures were used to estimate Body Mass Index (BMI) of Kg / m^2 of each child.

Actual motor competence assessment: The level of crude engine development was measured by the Test of Gross-Motor Development (TGMD) (Ulrich, 1985). TGMD is composed by 12-item test divided into loco motor (run, gallop, hop, leap, standing horizontal jump, slide) and control objects (strike, stationary ball bounce, catch, kick, overhand throw) sub tests. Three to four skill criteria are specific to each item and the operator must determine whether a child is proficient or not. For each item, the participants performed three tests; Before the evaluation started, participants participated in a low intensity startup program for ten minutes. For each test, the raw score allowed testing locomotives ranged from 0 to 26, while the raw score for controlling subjects controlled subjects ranged from 0 to 19. The raw score of each participant is converted into a benchmark for each test, individually, according to the age of each child. Next, the standard score was used to estimate Gross Motor Development Quotient (GMDQ). GMDQ has been used to explain the child's actual motor capacity level according to the normative data provided in GMDQ (i.e., very low, low, under the mean, mean, over the mean, high, very high). In the current study, four operators were involved in assessing the development of crude engines by means of GMDQ. The executives have followed the performance of each item in the gym, and they have recorded the performance videos of each child. After that, the evaluation of each test was performed by each operator, separately, with Longomtach software (LongoMatch, Ver. 0.20.8, http://longomatch.org) taking up some processes (e.g., : slow motion, moving videos forward and behind the frame on each frame) useful to provide a valid and reliable assessment of participants' performance. The reliability between the measurements ranges from 0.87 allowing the test of the machine to 0.91 to allow for control of the object.

Children's Recreational Activities Study Survey (CRASS): CRASS is a questionnaire used to investigate the children's extra-school daily routine in terms of physical and sport activities (i.e., 30 items like dance, tennis, soccer, to ride a bike, etc.) and recreational activities (i.e., 14 items like playing a musical instrument). For each activity, it is necessary to report the frequency (how many times from Monday to Friday and how many times on weekend) and the total time spent by the child in these activities (minutes or total hours from Monday to Friday and from Saturday to Sunday). The version of CRASS used in this study was the proxy-report, since the parents filled the questionnaire according to the activities performed by their child during the aforementioned periods. According to cultural and geographical location of the participants engaged in this study, some physical activities were selected and grouped together into three categories with homogeneous characteristics: sport, physical activity, and leisure. This procedure agreed with the one used in previous studies (Howley, 2001).

Moreover, for overcoming statistical problem related to a lot of structural zeroes obtained for many activities, the next step was to commute the time spent for each of the aforementioned three categories in dichotomous variables: a) from 0 to 120 minutes; b) more than 120 minutes. Finally, two levels of motion were defined according to the obtained time: a) Low Level of Motion (LLoM): participants were included in this category if their physical activity and sport time were from 0 to 120 minutes; b) High Level of Motion (HLoM): participants were included in this category if their physical activity and sport time were more than 120 minutes. It requires the involvement of a specialized expert in physical education, called "school sport tutor", who is appropriate trained for the project and works alongside the teacher for one hour a week, collaborating in planning and implementing motor activities.

As regard to the teaching-learning activities considered in this study, the tutor developed a teaching plan

following the Vietnamese National Guidelines for the development of the curriculum of physical education in primary school and he has carried out PE lessons based on games activities. These lessons included team and individual games and they differ from the sport-based training approach because the rules of the games are not codified, they do not cause early selection neither specialization, and small-sided games and circuit were used as prominent teaching approach. Circuits have been developed using available tools, such as cones, mats, over, balls, obstacles, and rope. The participants involved in this project were year 9 to 10 students and they followed the treatment for twenty weeks during the last school's year.

Data analysis: First, data related to motor competence were checked for verifying whether they were normally distributed or accounted for some univariate outliers. Then, according to the level of motion and gender, student's t tests for paired samples were conducted for verifying if exist a significant effect of the SdC project on the level of actual motor competence. If significant effect was found, the size of that effect was established by means of the non-clinical magnitude-based inference method (Hopkins et al., 2009).

The interpretation of the effects in a negative, trivial, or positive practical sense on the dependent variables (i.e., Locomotor score, Object control score, and GMDQ) was based on the following thresholds: <0.5 % most unlikely; 0.5-5% very unlikely; 5-25% unlikely; 25-75% possibly;75-95% likely; 95-99.5% very likely; and >99.5% most likely (Batterham & Hopkins, 2006). For each significant effect, we reported the t-value (t), the degrees of freedom (df), p-value, 90% confidence limits (CI90%), and the practical inference true effect, as suggested by Hopkins and colleagues (2009). The analyses were conducted using SPSS 20.0 and the alpha level was set to 0.05 in all tests.

Table 1. Describe children's anthropometric characteristics according to their movement level.

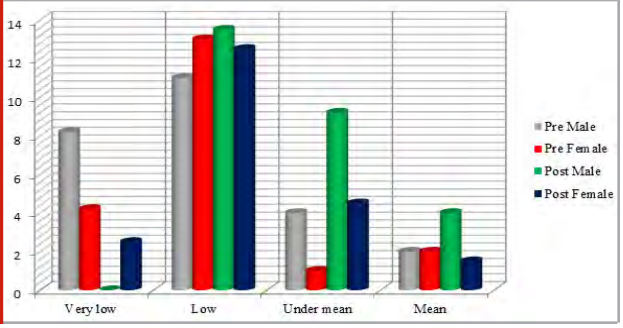
	High Level of Motion (n=30)		Low Level of Motion (n=32)	
	M	SD	M	SD
Age (year)	9.40	0.63	9.28	0.68
Height (m)	1.31	0.72	1.35	0.63
Weight (kg)	32.12	10.15	34.24	9.32
BMI (kg/m2)	1.78	0.42	1.84	0.31

RESULTS AND DISCUSSION

Data analysis results show that 12 subjects are not different, so their data has been removed from further analysis. Because no other violations were verified, parametric analyzes were performed. The further results are presented by following the analysis according to the participants' level of motion and, for each level, to participants' gender.

Participants with low level of motion: By considering the overall sample classified in the low level of motion, no significant differences were noted between the score pre- and post-project for the three parameters considered as proxy of the actual level of motor competence. If the gender was used as factor, significant effect of the lessons on motor competence was identified only for the male. In detail, significant differences were noted for: -) locomotor score (t= 3.45, df= 21, p=0.001, 90% confidence limits 2.0 to 5.2, most likely): the score related to these abilities was higher in the post- than in pre-lessons assessment; -) GMDQ (t= 1.98, df=22, p=0.05, 90% confidence limits 0.4 to 3.6, likely: the score used for quantifying the overall level of gross-motor skills development was higher in the post- than in pre-lessons assessment; The images in Figure n.1 show the influence of the treatment according to the GMDQ's aforementioned levels.

Figure 1: GM QD levels in low level of motion participants (LLoM) pre- and post-lessons Participants with high level of motion



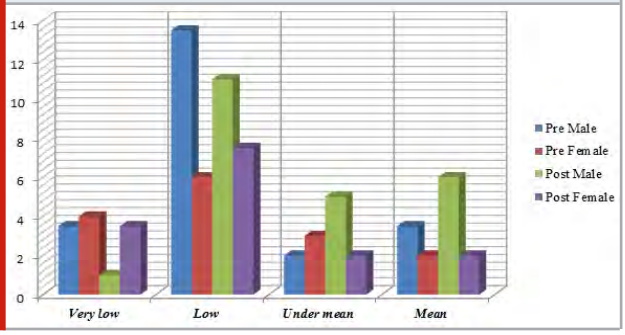
By considering the overall sample classified in the high level of motion, significant differences were noted between the score pre- and post-lessons for the following parameters: -) locomotor score (t= 3.32, df= 23, p=0.001, 90% confidence limits 2.0 to 5.4, most likely): the score related to these abilities was higher in the post- than in pre-lessons assessment; -) GMDQ (t= 2.09, df=23, p=0.03, 90% confidence limits 0.6 to 4.2, very likely: the score used for quantifying the overall level of gross-motor skills development was higher in the post- than in pre-lessons assessment; If the gender was used as factor, significant effect of the teaching-learning project on motor competence was identified only for the female. In detail, significant differences were noted for: -) locomotor score (t= 3.10, df= 13, p=0.001, 90% confidence limits 1.3 to 4.1, very likely): the score related to these abilities was higher in the post- than in pre-lessons assessment; The images in Figure n.2 show the influence of the treatment according to the GMDQ's aforementioned levels.

The purpose of this study was to evaluate whether the project "Sport di Classe" had an influence on the FMS development of Vietnamese children according to their level of motion and gender. Overall, the current results highlighted statistically significant positive differences in locomotion scores and GMDQ for the males. Girls showed a significant improvement only in locomotion

scores. No significant differences were found in object control scores. These results are in partial agreement with previous similar studies which have shown that male are more skilled than females. (Valentini et al., 2016; Sgrò et al. 2017). Other studies that have addressed the effect of physical activity interventions on fundamental movement skills underlined an improvement of such skills (Brusseau, et al., 2016).

Bryant and colleagues (2016) found that one day per week of physical education protocol performed by a class of Year 4 and Year 5 (experimental group) increased significantly mastery of fundamental movement skills, pedometer step and physical self-perception in comparison with a peer-age class enrolled in a game-based physical education process (control group). As regard to the evidences of the CRASS questionnaire and their relationship with the scores obtained in the GMDQ, the current result show that the males, who have been included in the Low Level of Motion group (LLoM) had an improvement more significant than the ones of the High Level of Motion (HLoM) group both in locomotion and in GMDQ scores.

Figure 2: GMQD levels in high level of motion participants (HLoM) pre- and post-lessons



A contrary effect it was estimated for the females: only the girls classified in the HLoM were affected by a significant and very likely positive effect of the treatment in their locomotion scores. In line with these results, the influence of the SdC project seems to be different according to gender, level of motion, and skills typologies and this evidence need to be considered for developing effective curriculum and teaching strategy. Results are similar to previous studies (Hardy et al., 2012). Developing FMS should be an important strategy in early childhood interventions aimed at promoting long-term physical activity, especially in elementary schools. Indeed, the appropriate development of FMS can affect the degree to which capabilities are implemented and this may affect the level of potential activity achieved in physical education activities (Fairclough & Stratton , 2005).

However, the overall level of gross motor development remains under the mean provided by Ulrich's normative data both in the assessment performed before and after the PE lessons, as shown in the figures 1 and 2.For this reason, the expectation is that children are more skilled than less experienced children. In fact, in the analysis of Hardy and colleagues, low scores in object control and mobility are related to inactive subjects. In a review written by Logan and colleagues (2015), studies have found a positive relationship between FMS and physical activity, except that the research was developed by Erwin and Castelli (2008).

So, "in accordance with the trends identified by Dordel (2000), as the decades passed, there is a lowering of the critical age where the children motor performances taking negatives features in respect with the past standard values" (Filippone et al.,2007). It can be assumed that this becomes worse, in the context of the relationship between physical activity and FMS, due to the dissatisfaction of the physical activity guidelines of children and adolescents.However, although there is growing evidence of the importance of FMS and its link to the physical and psychosocial development of children (Logan et al., 2012), the literature emphasized these capabilities in children and adolescents, (O'Brien et al., 2016).

The most worrisome feature is the warning that, over the generations, the worsening trend of engine power indicators is generally a change in age prediction.Current results are consistent with the aforementioned analysis, and they confirm that an on-site inspection protocol of physical activity is not sufficient to improve the level of FMS development to an appropriate score. Moreover, it has been verified that the development of FMS needs to be supported by the real physical education process based on student mobility and it needs to take into account the students and the gender of the sport, and the level of movement. However, the current results are affected by a number of limitations, such as low sample size and the lack of any useful factors to verify project effectiveness for cognitive learning areas, and social relationships. These factors need to be strongly considered planning for new studies with the aim of closely related to the studies mentioned in this study.

CONCLUSION

The main points of current and useful research for practitioners are: Low level of FMS development is still an important issue to face with all stakeholders in education plan. The effectiveness of physical education lessons is analyzed, although the relative positive results for the selected participants are not enough to overcome the aforementioned problems and to establish a heterogeneous effect to support a healthy lifestyle throughout life; Physical education goals can be achieved if they are supported, in elementary schools, with an effective curriculum based on well-known teaching strategies such as game-focused and tactical methods.

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The effect of Dry Season Stretch on Chlorophyll Content and RWC of Wheat Genotypes (*Triticum durum* L.)

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ABSTRACT

Drought stress is considered among the essential environmental stresses. Moreover, Wheatfields are under the danger of drought stress. In 2013, an experiment with the design of a randomized complete block (RCB) with 3 replications was performed in Ardabil Province, Iran, to assess the impact of drought stress on RWC (relative water content) as well as the chlorophyll content of the wheat genotypes. Six wheat varieties were evaluated in this study, which include Kavir, Sardari, Varinac (resistant varieties), Tajan, Marvdasht, and Ghods (susceptible varieties). At the germination stage, water was withheld to apply drought stress. The results showed the difference between susceptible and resistant genotypes in terms of RWC, chlorophyll content, K, and Na ions concentration. Thus, these measures may be used to screen wheat drought tolerance.

KEY WORDS: WHEAT, DROUGHT STRESS, RELATIVE WATER CONTENT, CHLOROPHYLL CONTENT, MINERAL ELEMENT.

INTRODUCTION

The chlorophyll content is among the important factors that impact the photosynthetic capacity. Drought stress may reduce or does not affect the plants' chlorophyll content in different plant species whose intensity is related to the intensity and duration of stress (Rensburg and Kruger, 1994; Kyparissis et al., 1995; Jagtap et al., 1998). The leaf chlorophyll content is an indicator of the photosynthetic capability of the tissues of plants (Nageswara et al., 2001; Wright et al., 1994). Flooding irrigation near one centimiter above the soil surface caused senescence and reduction in leaves' chlorophyll content. Schelmmer et al. (2005) reported that drought stress does not significantly affect maze leaf chlorophyll content. They concluded that turger pressure reduction due to water deficit alters the quantity of far-red light which crosses the leaf and thus changes the chlorophyll meter device measurements (Gholamin and Khayatnezhad 2020). Drought stress increment increased the light reflection from the leaf surface. Barry et al. (1995) reported a similar result for wheat. Moreover, Fotovat et al. (2007) demonstrated that severe drought stress would significantly reduce the chlorophyll content of wheat leaves.

In the mid-1980s, RWC was developed as the best criterion to show the water status of plant used afterward in place of water potential. RWC is related to the cell volume and thus may accurately indicate the water absorbance and consumption balance through transpiration. Schonfeld et al. (1988) proved that high RWC wheat cultivars show higher resistance when subjected to drought stress. In general, osmoregulation appears as a major mechanism to preserve the turgor pressure in the majority of plant species when facing water loss, which allows the plant to absorb

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water and continue its metabolic activities (Gunasekera and Berkowiz, 1992). Moreover, Zlatko Stoyanov (2005) showed that 14 days of drought stress until reaching a soil potential of -0.9 Mpa strongly decreased the turger pressure and osmotic potential in the first bean leaf. Ramos et al. (2003) demonstrated the significant RWC reduction in the bean leaves when facing drought stress. Lazacano-Ferrat and Lovat (1999) exerted drought stress to the bean plant and evaluated stem RWC 10, 14, and 18 days after withholding irrigation. They reported a significantly lower RWC in comparison to control plants. Gaballah et al. (2007) exerted anti transpirant matters on 2 Sesame cultivars, namely Shanavil 3 and Gize 32. They witnessed this matter via water, avoiding transpiration through the leaves, which resulted in an RWC increment in the mentioned cultivars.

No information is exists regarding the micronutrient spatial distributions in the grasses' raising leaves in drought conditions in addition to the comparative reactions of diverse species when subjected to salinity and drought stresses (Yuncai e al., 2007). The significant role of metal ions (paramagnetic ions) is well known in water binding in plants (Joseph et al., 1996). Potassium plays a vital role in water relations and stomatal activity (Marchner, 1995; Mengel and Kirkby, 2001). K+ presence in the plant reduces with diminishing soil water content since K+ mobility decreases in such conditions. The plants' capacity to keep high potassium concentrations in tissues appears to be a valuable characteristic to consider in the refining genotypes for the purpose of high resistance of drought stress. Recently, it has been found that intracellular Ca2+ regulates the plant's response to salinity and drought (Gholamin and Khayatnezhad 2020). Also, it has been demonstrated in the transduction of signals of salt- and drought-stress in plants, which play a crucial role in the osmoregulation in such conditions (Knight et al., 1997; Bartels and Sunker, 2005 Sallam et al 2019).

In many plants, high sodium level in an exterior solution reduces both Ca2+ and K+ tissue concentrations (Hu and Schmidhalter, 1997). This reduction may be attributed to Na+ and K+ antagonism at the roots uptake sites, Na+ effect on K+ transportation into the xylem (Lynch and Läunchli, 1984), or the uptake processes inhibition (Suhayda et al., 1990). Not much evidence is known regarding the drought impact on Mg in the plants. Hu and Schmidhalter (2005) reported that drought decreases the uptake of Mg.This study is intended to define chlorophyll content, RWC, and the mineral elements of the Wheat leaves when subjected to drought stress in Karaj, Iran.

MATERIAL AND METHODS

In 2008, to assess the impact of drought stress on the chlorophyll content, RWC (relative water content), and the mineral element of six Wheat genotypes, we conducted an experiment with three replications using randomized complete block design in Karaj, Iran. This study included six Wheat genotypes (Sardari, Kavir, Tajan, Varinac, Marvdasht, and Ghods). We exerted drought stress through water withholding at the anthesis stage. A chlorophyll meter device was used to measure the chlorophyll content. For RWC calculation, we weighted the Leaf fresh samples, then flooded the fresh leaves in distilled water and directly heated them for 48 h at 70 °C. We weighted the leaves again. Finally, we calcuated RWC based on Dhopte and Manuel (2002):

$$RWC = (FW\text{-}DW/TW\text{-}DW) \times 100$$

Where, FW is fresh weight, DW is dry weight and TW is turger weight of leaf samples. Na and K were determined by flame photometry (Eppendorf Flex 6361 model). Ca and Mg were determined by potentiometric titration with EDTA solution.

RESULTS AND DISCUSSION

Change of Leaf Chlorophyll: Drought stress significantly ($p<0.01$) affected the chlorophyll content of leaf genotypes (Table 1). The results of this study showed that the highest chlorophyll content belongs to resistant genotypes, and the Kavir genotype as a resistant genotype had significantly higher chlorophyll content (51.89 SPAD) under drought stress. Tajan and Ghods genotypes, as susceptible genotypes, had a significantly low chlorophyll content. Water deficit may damage chlorophyll and inhibit chlorophyll synthesis (Lessani and Mojtahedi, 2002).

Table1. Mean comparisons of effect of genotypes on measured trails in drought stress

Treatments	Chlorophyll content	RWC	K	Na	Ca	Mg
	Resistant genotypes					
Sardari	49.34b	74.43a	5a	4.16a	1.2a	0.26c
Kavir	51.89a	79.96a	5a	2.5c	0.7c	2.88a
Varinac	50.07b	72.2ab	5a	4.16a	0.66c	0.32c
	Susceptible genotypes					
Tajan	45.01d	64.3bc	3.75c	3.66ab	1b	0.56bc
Marvdasht	46.98c	73.2ab	2.5d	2.91bc	0.3d	0.92b
Ghods	44.26d	59.3c	3.9b	2.83c	1.13ab	0.29c

Moreover, a group of investigators have stated that leaf pigment damage caused by water deficit (Montagu and WOO, 1990; Nilsen and Orcutt, 1996). Mensah et al. (2006) exposed Sesames to drought stress and showed that it leads to increased leaf chlorophyll, which remains unchanged. Besides, Beeflink et al. (1985) revealed increased chlorophyll content in onion when subjected to drought stress. Water deficit may reduce chlorophyll content through heat or drought stress through producing ROS (reactive oxygen species), including H2O2 and O2-, which may cause lipid peroxidation and hence, chlorophyll damage (Mirnoff, 1993; Foyer et al., 1994). Similarly, reduced chlorophyll content caused by the change of the leaf's green color into yellow leads to an increment of the incident radiation reflectance (Schelmmer et al., 2005 Sallam et al 2019). Apparently, the mentioned mechanism may guard the photosynthetic system when facing stress. Lawlor and Cornic's (2002) study showed that reducing carbon assimilation, which confronts water deficit led to the destruction of photosystem 2 D1 protein (Xian-He et al., 1995) without any currently known explanation.

RWC was significantly affected by drought on the genotypes ($p<0.01$) (Table 1). The highest values belonged to Sardari and Kavir genotypes with 74.43 and 79.96%, respectively, while the lowest RWC belonged to Ghods genotype with 59.3%. Leaf RWC is among the best biochemical/ growth indexes, which show the severity of stress (Alizade, 2002). The RWC rate in highly resistant plants against drought is above other plants. Alternatively, plants with higher yields when subjected to drought stress require to have a high RWC. Thus, according to the results of this study, the mentioned genotypes, classified as genotypes with high and medium yield when subjected to drought stress, would show higher RWC. Plant RWC reduction when subjected to drought stress depends on its vigor decrement, which is the case in many plants (Liu et al., 2002). In the case of water deficit, the cell membrane is vulnerable to alterations including reduced sustainability and penetrability (Blokina et al., 2003). A microscopic study of dehydrated cells showed injuries such as cell membrane cleavage and cytoplasm content sedimentation (Blackman et al., 1995). Possibly, under such states, osmotic adjustability is decreased (Meyer and Boyer, 1981). In this case, it appears that the concentration of proper solutes is not enough to maintain the membrane.

Change of Mineral Elements: The current paper indicated that the varying of the mineral element among genotypes when subjected to drought stress (Table 1). Accordingly, tolerant genotypes provided the maximum potassium concentration, while susceptible genotypes provided the maximum sodium concentration. Though Ca and Mg differences were not significant between susceptible and resistant genotypes. K and Mg deficiency may lead to a considerable reduction in the metabolism of photosynthetic C as well as fixed carbon utilization (Mengel and Kirkby, 2001). Due to the distinctive impacts of K and Mg on the photo-oxidative damage in the plants which are matured in marginal conditions, including chilling, salinity, and drought may be worsened in the case of low K or Mg soil supply. The sufficient potasium supply beneficial effect was attributed to the K role in photoassimilates retranslocation in roots, which caused superior root growth when subjected to drought stress (Egilla et al., 2001). Considering these results, we can attribute the K protective effects in drought stress to its inhibitory effects, which makes the plants more sensitive to the drought stress.

CONCLUSION

Crop plants' productivity and survival depend on their exposure to environmental stresses and their adaptive mechanisms in order to prevent or tolerate stress. Mounting evidence shows that the plant's mineral nutritional status considerably impacts its adaptation ability when subjected to hostile environmental conditions. In the current study, we discussed the effect of the mineral nutritional status of the tolerant genotype in adapting to a state of drought stress. The present study was intended to investigate the characteristics of resistant plants against drought stress. Results of the current study demonstrated that RWC, chlorophyll content, the concentration of K, and Na ions were different between susceptible and resistant genotypes. Hence, these measures may be utilized as a screening tool to assess Wheat drought tolerance.

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Reference Evapotranspiration Calculation in the Zone of Ukrainian Polisia Using Air Temperature

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ABSTRACT

Reference evapotranspiration is an important index for deep evaluation of climate conditions in climatology and irrigation requirements in agriculture. Current standard method of Penman-Monteith supported by Food and Agricultural Organization, FAO has proved its high reliability and accuracy in most areas of the world. However, very often it is impossible to derive reference evapotranspiration using the mentioned method because of lack of some necessary data for accurate computation. The goal of the study was to derive the reference evapotranspiration in Ukrainian Polisia for the warm period of year by the air temperature as the only input required for the calculation through the creation of regression equations, which were developed based on the linear regression analysis at $p<0.05$ of 488 meteorological samples of the reference evapotranspiration in the area of the study calculated by the standard FAO method in ET_0 Calculator software. The results of the study proved the possibility of rough and fast estimation of the reference evapotranspiration in Ukrainian Polisia using air temperature only with the mean average percentage error (MAPE) of the computation at the level of 30.10%. The values of MAPE ranged by the oblasts of the area of the study within 26.03–29.34% with the minimum error for Chernihiv, and the maximum – for Lutsk (Volyn) oblasts of Ukraine.

KEY WORDS: AIR TEMPERATURE, PENMAN-MONTEITH ALTERNATIVE, REFERENCE EVAPOTRANSPIRATION, REGRESSION ANALYSIS, UKRAINIAN POLISIA.

INTRODUCTION

Reference evapotranspiration (ET_0) is a valuable index for determination of demands for irrigation water and climate parameters (Hargreaves, 1994). The technique of ET_0 estimation embraces direct (mainly lysimetric) and indirect methods. Indirect methods are represented by a set of formulas, based on empirical description of physical processes, which take place in the system "plant-soil-atmosphere" and are connected with transport of water during physiological processes in plants in the concrete weather conditions. The most wide-spread indirect method, which is generally accepted as the standard worldwide, is the method of Penman-Monteith (Sentelhas et al., 2010). Notwithstanding the fact that this method was proved to be the most reliable in the estimation of ET0, it is not possible to apply it in every study due to the difficulties, connected with sophisticated computations and huge amount of the input data required to obtain a reliable result.

FAO provides software named ET_0 Calculator, which is aimed to simplify the process of reference evapotranspiration assessment by Penman-Monteith method; however, it is still advisable to provide all the inputs to get the correct estimation, although it is possible to come to nothing

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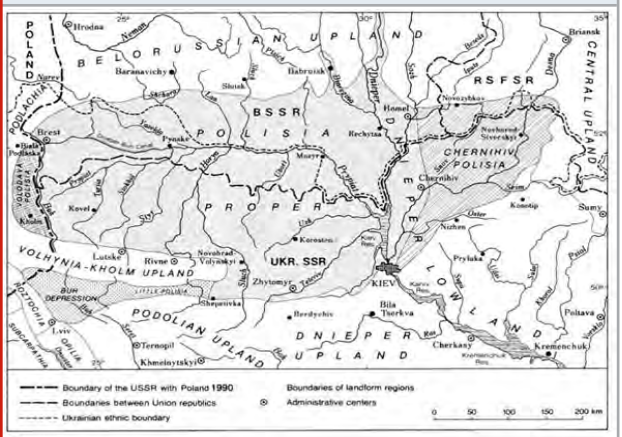
more than the maximum and minimum air temperature to perform the rough assessment (Raes & Munoz, 2009). The previous study of Lykhovyd, (2020) has proved that the idea is not only theoretically interesting, but is of practical value and provides a good chance for agricultural producers to estimate and forecast ET0 in the concrete zones using fast and convenient formula of K×T (K – coefficient, mm/°C; T – air temperature, °C, (Lykhovyd, 2020). The goal of the study is to provide an alternative method for local ET0 computation using the most common weather index – air temperature – as the only input for the reference evapotranspiration estimation in the zone of Ukrainian Polisia.

MATERIAL AND METHODS

Polisia is one of the largest forest zones of Europe and it is located in the south-western part of the Eastern-European Lowland, or in the Polisia Lowland. Ukrainian Polisia is the part of the natural zone, which occupies mainly Northern Ukraine, namely, Volyn (Lutsk), Rivne, Zhytomyr, Kyiv and Chernihiv Oblasts (Internet Encyclopedia of Ukraine). Therefore, the main big cities and meteorological stations situated in the zone are in Lutsk, Rivne, Zhytomyr, Kyiv and Chernihiv (Fig. 1).

Figure 1: Ukrainian Polisia on the map of Nothern Ukraine, Eastern Poland, Southern Belorussia, and Western Russia (Internet Encyclopedia of Ukraine)



The study was based on the long-term meteorological data on monthly maximum, mean, minimum air temperature and wind speed during the warm period (average air temperature ≥0°C), which were obtained from Lutsk, Rivne, Zhytomyr, Kyiv and Chernihiv hydrometeorological stations for the period of January 2010 – October 2020. The mentioned stations were chosen as the key ones to represent the zone of Ukrainian Polisia. The total number of data inputs was: Volyn (Lutsk) oblast – 76; Rivne – 105; Zhytomyr – 102; Kyiv – 105; Chernihiv – 100, Ukrainian Polisia overall – 488. Meteorological indices were used as the inputs for calculation of the reference evapotranspiration using Penman-Monteith equation in ET_0 Calculator software (the developer – FAO UNO). The pairs of "mean air temperature – ET_0" were formed and processed by linear regression within Biostat v7 software at $p<0.05$. To evaluate the accuracy of the ET_0 computation using the developed regression models the values of mean absolute percentage error (MAPE) and coefficient of determination (Predicted R^2) were estimated for each zone (Moreno et al., 2013).

RESULTS AND DISCUSSION

As a result of regression analysis, six models for reference evapotranspiration computation based on the air temperature only were developed, including not only the model for Ukrainian Polisia overall, but for every oblast of this zone (Table 1).

Table 1. Reference evapotranspiration calculation for Ukrainian Polisia and its sub-zones using air temperature (AT), ± MAPE

Name of the zone	Calculation equation (Predicted R^2)
Lutsk (Volyn) oblast	0.3551×AT±29.34% (0.9432)
Rivne oblast	0.4122×AT±28.18% (0.9255)
Zhytomyr oblast	0.2799×AT±26.83% (0.9484)
Kyiv oblast	0.3179×AT±26.79% (0.9459)
Chernihiv oblast	0.3508×AT±26.03% (0.9391)
Ukrainian Polisia	0.3410×AT±30.10% (0.9235)

The values of MAPE and Predicted R^2 testify about acceptable accuracy of the developed regression models for fast and rough estimation of the reference evapotranspiration in the zone of Ukrainian Polisia and some Northern oblasts of Ukraine. The best accuracy was obtained for Zhytomyr, Kyiv, and Chernihiv oblasts, while the overall result for Ukrainian Polisia is slightly worse in accuracy. The highest reference evapotranspiration with accordance to the regression model coefficients is usual for Rivne oblast (mainly owing to strong winds in the region), while the least evapotranspiration is associated with Zhytomyr oblast (light winds and favorable temperature regime is a feature of this region).

Our study is just another option among the available ones for reference evapotranspiration estimation in case of lack of required for full Penman-Monteith equation meteorological data. While some scientists look upon remote sensing technologies as a relevant and most applicable alternative in this case (Cammaleri & Ciraolo, 2013), others try to apply weather forecasts data instead of the data derived directly from meteorological stations (Er-Raki et al., 2010), or are looking for the simpler available method testing the accuracy in comparison with Penman-Monteith one (Tabari et al., 2013), our study was directed on simplification of the original ET_0 calculation equation by the methods of mathematical statistics. The application of mathematical statistics in the form of artificial neural networks has been proved to be quite effective for the purpose (Feng et al., 2016).

Although neural networks are considered to be the best option in such cases, providing the highest accuracy in life sciences (Lykhovyd, 2018), our study was focused on much simpler technique of linear regression analysis, which might be less accurate but more practical as it is possible to get the result in the form of an equation. Besides, it has been proved by the results of our previous study on the reference evapotranspiration estimation in the South of Ukraine that the accuracy of such an approach is sufficient enough to be used in science and practice of irrigated agriculture for rough estimation and forecast of reference evapotranspiration (Lykhovyd, 2020).

CONCLUSION

It is possible to assess reference evapotranspiration in the zone of Ukrainian Polisia by the formula $ET_0 = 0.3410 \times AT$. The accuracy of the method is 30.10%. The mentioned model is recommended for reference evapotranspiration estimation in the zone both in practical and scientific purposes. The model is going to be used in the complex research aimed to provide general ET0 computation formula for Ukraine. Further studies on the enhancement of the model quality are also projected.

Conflict of Interest Statement: There is no conflict of interest regarding the publication of this paper.

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Oral Health Knowledge and Attitude Among Students of King Abdulaziz University Jeddah Saudi Arabia

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ABSTRACT

Maintenance of oral health and positive attitudes has an integral role in the success of dental treatment and has a direct effect on the prognosis. This study aimed to determine if dentists have more dedication to their oral health than non-dentists, as there is limited data surrounding this topic in Saudi Arabia. An electronic survey was conducted to collect the data from anonymous participants in both dental and non-dental fields. Main contents of survey questions were brushing, flossing, washing, fillings, crowns, extractions, orthodontics, halitosis, gum status, toothache and checkups. Chi square and Monto Carlo tests were used to analyze the results which detected significant differences on extractions, orthodontic appliance, halitosis and gum status for dental students with corresponding scores of (18.9%, 64.9%,16.2%, 20.3% respectively) whereas non-dental students scored (33.6%, 45.8%, 29.8%, 40.5% respectively). With P-value of 0.025 for extractions, 0.009 for orthodontic appliance, 0.031 for halitosis, and 0.006 for gum status. The most prevalent problem was orthodontics. A significant difference was found between dental and non-dental students in terms of tooth brushing and flossing (75.7% VS 55.8% and 71.5% VS 23% respectively) (p = 0.046, P< 0,001 respectively). Missing tooth and halitosis were higher among males than females (50% VS 25.3% and 50% VS 22% respectively) with significant difference (p=0.011, 0.003 respectively). we highly recommend more oral hygiene health education programs especially for non-dental students. Further research is needed to assess oral health problems based on clinical examination and comprehensive detailed interviews to overcome internal validity errors that might occur in an electronic based survey.

KEY WORDS: DENTAL, ORAL, HEALTH, ATTITUDE, SAUDI ARABIA.

INTRODUCTION

Attitudes of dental students towards oral hygiene affect their habits and help improvements of oral health of their society and patients (Sasanka et al., 2020). Good oral health depends on proper oral hygiene which helps in improving the quality of life (Halawany, 2012). It affects appearance, allows people to perform their daily activities without psychological or social limitations (Shah and El Haddad, 2015). Oral health relies on many factors such as personal attitudes, behaviors and knowledge. Dentists should be a role model for oral health attitudes and practices to their communities (Mekhemar et al., 2020). Dentists have a great role in this by providing prevention, treatment and helping people change their behavior and attitudes towards oral hygiene (Moheet and Farooq, 2013). However, poor oral health awareness was reported among Saudi dental students (Baseer and

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Rahman, 2014). In addition, gingival diseases were found to be highly prevalent among dental students (Ahmad et al., 2019). There is an observed need to increase oral health awareness, positive attitudes and correct practices of dental students (Ahmad et al., 2019). Therefore, teaching students the skills needed for good oral health is as important as giving them knowledge (Halawany et al., 2015). In literature, the focus was mainly on treatment of the oral diseases rather than prevention, (Levin and Shenkman, 2004, Glick et al., 2012, Al-Nasser and Lamster, 2020).

The aims of this study are to increase awareness of oral hygiene importance to both dental and non-dental students, determine the most prevalent oral health problems among the groups in order to formulate a targeted community service. This in addition to assessing oral hygiene, attitudes and compliance among dental students and non-dental students and among female dental students or male ones in King Abdul-Aziz University Jeddah Saudi Arabia.

MATERIAL AND METHODS

This comparative cross-sectional study included 206 students at King Abdul-Aziz University Jeddah KSA. Electronic database search was done using Google Scholar and PubMed to gather background information and data related to the research question and determine the gap of knowledge. Then, formulating an anonymous electronic survey with multiple choice questions for dental and non-dental students to answer during the period from 29/01/2020 to 31/08/2020, followed by data analysis and segregation of the results. Arabic language was chosen for this survey to avoid any possibilities of language barriers, followed by data analysis and segregation from the survey. Students were stratified according to specialty and gender. Data were analyzed using the statistical package for the social sciences (SPSS), version 25. Categorical variables were presented as numbers and percentage. Results were compared using Chi-square test, Monto Carlo test and Fisher Exact test. All tests were 2-tailed, and a p-value of <0.05 was considered statistically significant.

Table 1. Oral hygiene and oral health according to dental specialty (n = 206)

		Dental students (n=74)		Non dental students (n=131)		P-value
		N	%	N	%	
Brushing	Not daily	3	4.1%	9	7.0%	0.046*
	Once	9	12.2%	28	21.7%	
	Twice	56	75.7%	72	55.8%	
	More than twice	6	8.1%	20	15.5%	
Floss	I don't	17	23.0%	93	71.5%	<0.001*
	Once	51	68.9%	29	22.3%	
	Twice	4	5.4%	6	4.6%	
	More than twice	2	2.7%	2	1.5%	
Missing tooth	I didn't	60	81.1%	87	66.4%	0.058**
	1-2	12	16.2%	28	21.4%	
	3-4	1	1.4%	13	9.9%	
	5-6	0	0.0%	2	1.5%	
	>6	1	1.4%	1	0.8%	
Orthodontics	Yes , currently	6	8.1%	11	8.4%	0.022*
	Yes, in the past	42	56.8%	49	37.4%	
	Never	26	35.1%	71	54.2%	
Halitosis	Yes	11	14.9%	39	29.8%	0.016**
	No	62	83.8%	92	70.2%	
Gum	Excellent	57	77.0%	72	55.0%	0.006**
	Bleeding during brushing	15	20.3%	53	40.5%	
	Bleeding mostly	0	0.0%	3	2.3%	

Note. All variables are summarized as number and percentage
The test of significance was carried out at 0.05 level
*Chi- Square test was used
**Monto Carlo test was used
Significant results are in bold

Ethical considerations: the study was approved by Research Ethics Committee of the Faculty of Dentistry (REC-FD) Consent was governed automatically by participating in the survey, as it was declared before taking it.

RESULTS AND DISCUSSION

Out of 206 participants, 88.3% were females, (86.4%) with average age of 20-30 years old. Descriptive data are shown in table 1 with 73.3% postgraduate and 63.6% were non dental students. In regard of oral hygiene measures, a significant difference was found between dental and non-dental students in terms of tooth brushing and flossing (p = 0.046, P< 0,001 respectively). However, no significant difference was found between dental and non-dental students comparing the oral and tooth conditions. In regards of dental visits and compliance, the dental students showed higher compliance than the non-dental students (Table 1).

The most common oral health problem among both dental (64.9%) and non- dental students (45.8%) was malocclusion followed by bleeding on brushing (p= 0.009 and p=0.006 respectively) (Table 2, fig.1).

Table 2. Prevalence of the most common Oral health problems in dental students and non-dental students (n=205)

	Dental students (n=74)	Non dental students (n=131)	P-value
Fillings	82.4%	75.6%	0.254*
Crowns	13.5%	9.9%	0.434*
Missing tooth	18.9%	33.6%	0.025*
Orthodontics	64.9%	45.8%	0.009*
Halitosis	16.2%	29.8%	0.031*
Bleeding during brushing	20.3%	40.5%	0.006**
Bleeding mostly	0.0%	2.3%	
Receding gum	2.7%	2.3%	
Toothache	17.6%	26.7%	0.137*

Note. All variables are summarized as percentage
The test of significance was carried out at 0.05 level
*Chi- Square test was used
**Monto Carlo test was used
Significant results are in bold

When comparing between both genders, missing tooth and halitosis were higher among males than females (50% VS 25.3% and 50% VS 22%) with significant difference (p=0.011, 0.003) (Table 3, fig 2).

This comparative cross-sectional study was conducted at King Abdul-Aziz University including 206 dental specialty and non-dental specialty students. This study assesses the knowledge and attitudes towards oral Health among Dental Students and Non-dental Students. To summarize our findings, dental specialty students had better attitudes towards oral hygiene and health, having less oral health problems than non-dental specialty students. Female students also had better oral attitudes with less prevalence of oral problems than males. As regard to oral health and attitudes among dental specialty students, our findings supported the conclusions of Gufran and colleagues. Most of our dental students reported brushing their teeth twice daily. In 2015, Gufran and fellows reported that 82% of their subject students brushed their teeth twice daily (Gufran et al., 2015).

Most of our students who reported not brushing their teeth on a daily basis were of a non-dental specialty. In addition, flossing was higher among dental students suggesting better oral health and attitudes among them. This corresponds to the findings of Santhosh Kumar et al., who found that dental students had a better attitude towards oral health and a better behavior when compared to pharmacy students (S. Kumar et al., 2012). Also another study showed that dental students had better knowledge, attitude and practice than medical students (H. Kumar et al., 2017).

In addition, a study by Baseer found a significant difference between dental hygiene behaviors among government and private universities students which was supported with a difference between the clinical and preclinical dental education (Baseer et al., 2013). In fact, knowledge among dental students is not satisfactory and they need more awareness about oral self-hygiene practice which is the same as what a study by Lavanya et al. concluded (Lavanya & Nallamilli, 2014).Regarding the prevalence of oral health problems in dental students and non-dental students, missing tooth, halitosis, bleeding on brushing and spontaneous bleeding were more frequently reported among non-dental students. This collaborates what Ahmad and colleagues reported in 2019; a study that documented a high prevalence of gingival diseases among all study subjects (Ahmad et al., 2019).

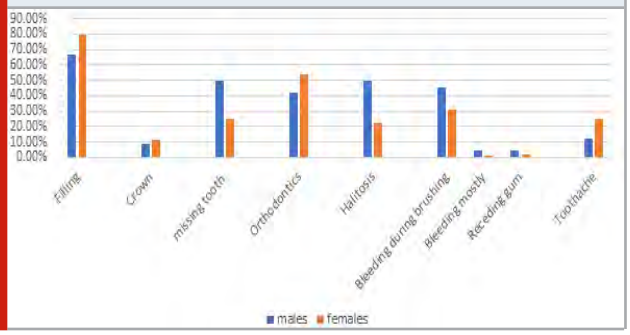
On the other hand, our study reported an unexpected higher prevalence of orthodontics among students of dental specialty. Similarly a study by Wagle et al. concluded that dentists had better oral health than general population and that the prevalence of decayed and unfilled teeth was lower among dentists, (Wagle et al., 2014).When it comes to gender-based oral health comparison among our sample, the prevalence rates of not brushing teeth daily, not using floss and halitosis were higher among males than females. Furthermore, the prevalence rates of missing tooth and halitosis were higher among male subjects than females. Therefore, we can conclude that females are more committed to oral health and hygiene. This could be a result of their concern about how their mouth and teeth look. This was similar to the findings of a study by Kumar et al. where they found that females had better knowledge and oral health practices than males (Kumar et al., 2017).

Table 3. Oral Health and attitude in dental students according to gender (n = 74)

		Males (N=24)		Females (N=182)		P-value
		N	%	N	%	
Brushing	Not daily	5	21.7%	7	3.9%	0.006*
	Once	5	21.7%	32	17.7%	
	Twice	11	47.8%	118	65.2%	
	More than twice	2	8.7%	24	13.3%	
Floss	I don't	19	79.2%	92	50.8%	0.072*
	Once	5	20.8%	75	41.4%	
	Twice	0	0.0%	10	5.5%	
	More than twice	0	0.0%	4	2.2%	
Missing tooth	I didn't	12	50.0%	136	74.7%	0.064*
	1-2	8	33.3%	32	17.6%	
	3-4	3	12.5%	11	6.0%	
	5-6	0	0.0%	2	1.1%	
	>6	1	4.2%	1	0.5%	
Orthodontics	Yes, currently	3	12.5%	14	7.7%	0.267**
	Yes, in the past	7	29.2%	84	46.2%	
	Never	14	58.3%	84	46.2%	
Halitosis	Yes	12	50.0%	39	21.4%	0.030*
	No	12	50.0%	142	78.0%	
Gum	Excellent	11	45.8%	119	65.4%	0.205*
	Bleeding during brushing	11	45.8%	57	31.3%	
	Bleeding mostly	1	4.2%	2	1.1%	
	No	21	87.5%	136	74.7%	
Checkup	Yes, every 6 months	3	12.5%	42	23.2%	0.689*
	Yes, once per year	5	20.8%	28	15.5%	
	No, only during pain	13	54.2%	91	50.3%	
	Never	3	12.5%	20	11.0%	

Note. All variables are summarized as number and percentage
The test of significance was carried out at 0.05 level
*Monto Carlo test was used
**Fisher exact test was used
Significant results are in bold

Figure 2: Prevalence of the most common Oral health problem according to gender.



Similarly another study by Jaber et al. found that male students had good knowledge but poor practice toward oral health, (Jaber et al., 2017). Our paper showed the difference in oral health and attitudes, prevalence of oral problems among dental and non-dental students, female and male dental students. Thus, we highly recommend more oral hygiene health education programs especially for non-dental students.

We obtained our data using an online survey which has some limitations such as possibility of inaccurate information as the survey is in the form of multiple-choice questions.

The survey could be answered by the same person twice as it was anonymous which could affect the integrity of the results. Limited number of questions were used as increasing the questions might bore the participant which could affect the accuracy of the results. Non-dental students might provide us with non-accurate answers because they are not keen in the dental field. Dental and

non-dental students could be biased either to their side or against which could affect the integrity of the results. Respondents may not feel comfortable providing answers that present themselves in an unfavorable manner.

CONCLUSION

Dental specialty students had better attitude towards oral hygiene, oral health and having less oral health problems than non-dental specialty students, so we highly recommend more oral hygiene health education programs especially for non-dental students. Female students were showing more positive attitude than males with the prevalence of oral health problems were more among male students. During literature search, we found limited data on oral health and attitudes of dental students in Saudi Arabia. About eleven studies conducted in Saudi Arabia were found, none of them was in Jeddah or King Abdulaziz University specifically. Further research is needed to assess oral health problems based on clinical examination and comprehensive detailed interviews to overcome internal validity errors that might occur in an electronic based survey.

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The State of Cardiac Activity in Greco-Roman Wrestlers on the Background of Different Options for Weight Loss

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ABSTRACT

The study involved 58 the Greco-Roman style (17-22 years) and four weight categories, with qualification not below the 1st sport degree. Registration of the heart was performed using electrocardiography and echocardiography in the precompetitive, competitive and postderegulation period. The athletes that used in precompetitive period, methodology, rational weight loss, have been identified in the competitive period of the change of heart, which is the options rules the "sports heart" (sinus arrhythmia, sinus bradycardia, atrioventricular blockage of I-th degree, and left ventricular hypertrophy) or of small anomalies of the heart (mitral valve prolapse with regurgitation of I degree), which requires medical monitoring but are not contraindications to sports activities. In post deregulation period primatologic hesky changes in the hearts of these athletes have been identified. The athletes, used in the precompetitive period of extreme methods of weight loss, in the competitive period revealed sinus tachycardia, arrhythmia, and arrhythmia, progressive post deregulation in the period, up to the maladjustment of the heart. Using methods of extreme weight loss in precompetitive period, the Greco-Roman style primatologichesky leads to changes of heart and should be reconsidered, in favor of rational techniques, based on manifested morpho-functional changes in the heart.

KEY WORDS: HEART, WEIGHT LOSS, GRECO-ROMAN WRESTLING, PHYSICAL EXERCISE, COMPETITION.

INTRODUCTION

Regular dosed physical activity provides the development of a number of regular changes in the human body (Stepanova et al., 2018). Under the condition of gradual and feasible increase in load, they are positive and provide for the strengthening of the cardiovascular and respiratory systems, as well as optimize microcirculation processes (Zavalishina, 2018a; Vorobyeva et al., 2018). All this contributes to the improvement of adaptation processes in the body of athletes and athletes. This is especially true in various martial arts (Magda, Afifi, 2017).The constantly increasing functional component in the classical struggle makes great demands on the cardiorespiratory system, the musculoskeletal system and the psychological state of athletes. However, the reserves for further increase in training loads are almost exhausted (Afonina, 2013; Chichkova, Svetlichkina, 2017). A promising way to increase the level of fitness can be to improve the means of training used, create more rational ratios of the volume and intensity of the load, and use more advanced forms of recovery (Andrade et al., 2014; Svetlichkina, Kozlyatnikov, 2016).

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Insufficient recovery of the wrestler's body leads to a decrease in its functional capabilities, contributes to the appearance of pathological phenomena in it, and causes the development of progressive chronic diseases (Karpov et al., 2019a).

The characteristic dynamism of competitive activity against the background of high emotional stress makes special demands on the cardiovascular system of athletes, and also requires the maximum mobilization of all functional reserves of other systems of their body (Karpov et al., 2019b). In many cases, competitive activity is carried out against the background of an extreme decrease in the body weight of athletes, which greatly increases the risk of developing pathology of the cardiovascular system (Boldov et al., 2018). In this regard, studies of the influence of physical activity of the competitive period on the state of cardiac activity in Greco-Roman style wrestlers, often resorting to an emergency way to reduce body weight, are relevant (Chichkova and Svetlichkina, 2017).The purpose of the study was to determine changes in the morphological and functional characteristics of the heart under the influence of physical activity in qualified Greco-Roman style wrestlers resorting to weight loss.

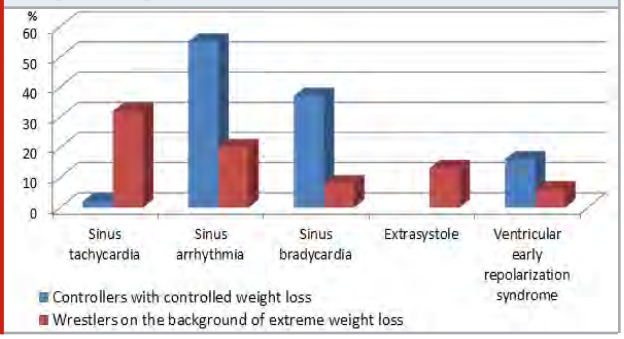
MATERIAL AND METDODS

A survey was conducted of 58 young men 17-22 years old (weight categories 55-60 kg; 60-63 kg; 77-82 kg; 82-87 kg, engaged in Greco-Roman wrestling, having qualifications from 1 sports category to the title of master of sports. All examined well-known emergency methods of weight loss before the competition. Their clinical data and indicators of their instrumental examination were analyzed. The studies were carried out using a 3-channel Cardiovit AT-101 electrocardiograph (Schiller, Switzerland), Sono line G60 S ultrasound apparatus (Siemens Ge romania), Microvit MT-101 Holter electrocardiography recorder and MT-200 analysis program (Schiller, Switzerland), Microsoft Excel 2007 spreadsheet programs with the macro add-on XLSTAT – Pro (Microsoft, USA).Statistical analysis of the obtained data was carried out by Statistica10 software package for statistical analysis (StatSoftInc., USA). The data were processed using the Mann – Whitney U-test by evaluating the correlation using the Spearman rank correlation coefficient.

RESULTS AND DISCUSSION

All subjects subjectively felt healthy, any complaints on the state of health was absent, auscultatory picture of the lungs in all cases were without pathology, pathological accents and a heart murmur over the heart area was tapped in 5 of the surveyed wrestlers (1 - a candidate master of sports, 4 masters of sports). The analysis of indicators of the electrocardiogram in athletes using methods for rational reduction of body weight during the precompetitive period revealed that at 55.8±5.2% of cases were detected sinus arrhythmia, at 37.2±7.1% of sinus bradycardia, which was regarded as a normal variant and served as an indicator of fitness athletes. At 16.2±2.5% of the wrestlers were was a syndrome of early repolarization of the ventricles as a result of changes in the autonomic nervous system with predominance of the influence of the vagus nerve, which was confirmed by the tests with physical load, in which the symptoms disappeared. In 23.2±3.1% of the surveyed wrestlers were registered atrioventricular block I degree (the interval PQ-0,21s), which is also considered a manifestation of "sports heart" and a good fitness of the cardiovascular system, but required in-depth surveys and dynamic control. In 69.7±2.5% of the surveyed wrestlers was hypertrophy of the left ventricle, which also require dynamic monitoring and control through echocardioscopy.

Figure 1: The frequency of occurrence of functional disorders and electrocardiograms of fighters in the pre-competition period.



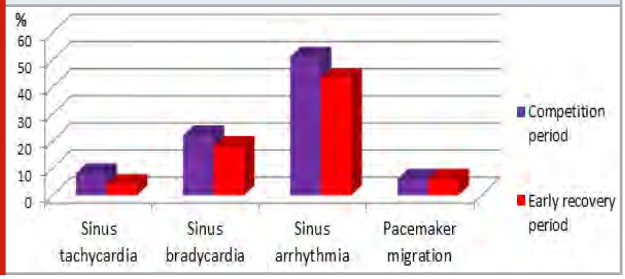
Auscultation of the 5 athletes (1 - a candidate master of sports, 4 masters of sports) auditioned noise mitral regurgitation that was confirmed by ultrasound examination of the heart, where were visualized mitral valve prolapse I degree with mitral regurgitation of I degree. These changes are attributed to small anomalies of heart development that are not contraindications to sports, but requiring control and dynamic examination at least 1 time a year for early detection of progression and transition these changes in the pathological condition. Athletes belonging to the weight category 55-60 kg and 60-63 kg, who resorted to extreme weight loss in preparation for the competition, recorded a number of characteristic changes in the electrocardiogram (Figure 1). In 33.3±3.7% of athletes using extreme weight loss techniques, sinus tachycardia ($\chi 2 = 8.5$; $p<0.005$; r - 0.577) was recorded, in 20.1±4.1% sinus arrhythmia ($\chi 2 = 4.1$; $p<0.005$; r - 0.632). In 13.3±3.3% of cases, a single supraventricular extrasystole was recorded ($\chi 2 = 2.4$; $p<0.05$; r - 0.421), which increases after a functional test with physical activity (recording an electrocardiogram after 30 squats).

The analysis of the electrocardiogram indicators in the competitive period revealed that the influence of the competitive load, as a rule, of maximum intensity, also affected the electrocardiogram indicators (Svetlichkina, Dorontsev, 2016) (Figure 2). Sinus tachycardia and tachyarrhythmia were recorded in 33.3±4.0% ($\chi 2 = 11.9$; $p<0.005$; r - 0.597) and 46.6 ± 5.3% ($\chi 2 = 7.7$; $p<0.005$; r - 0.509) athletes respectively.

Migration of the supraventricular pacemaker occurred in 20.1±3.2% ($\chi 2 = 9.0$; $p<0.005$; r - 0.711) cases. In 40 ± 5.5% ($\chi 2 = 5.4$; $p<0.005$; r-0.562) of the subjects, supraventricular extrasystole was recorded by the type of bigeminia and trigeminia against the background of sinus tachyarrhythmia.

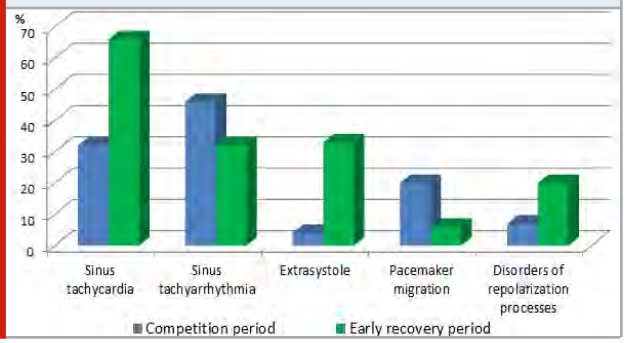
An analysis of the electrocardiogram indices of the same athletes in the post-competitive period, namely during the early recovery period, revealed that pathological changes were recorded much more often, which indicates disadaptation of their cardiovascular system (Svetlichkina et al., 2016; Legotkin, Lopatin, 2016). In 66.6 ± 5.2% of cases, sinus tachycardia was recorded ($\chi 2 = 9.3$; $p<0.005$; r - 0.592), in 47.3±3.7% sinus tachyarrhythmia was detected ($\chi 2 = 6.9$; $p <0.005$; r - 0.672). In 33.3 ± 2.9% of the subjects, supraventricular extrasystole ($\chi 2 = 5.3$; $p<0.005$; r - 0.517) by type of bigeminia and trigeminia was recorded, migration of the supraventricular driver ($\chi 2 = 2.4$; $p<0.005$; r - 0.412) rhythm was detected in 6% of athletes. Violation of the processes of myocardial repolarization in the lower wall ($\chi 2 = 7.2$; p 70.005; r - 0.519) were detected in 20% of wrestlers, which was associated with the presence of stressful cardiomyopathy against the background of physical overload. Athletes of groups of weight categories 77-82 kg and 82-87 kg, using methods of planned regulation of body weight, did not reveal a critical decrease in reserves and pre-pathological changes in the cardiovascular system in the competitive and post-harvest periods (Figure 3).

Figure 2: The frequency of occurrence of functional disorders and the electrocardiogram of wrestlers against a background of controlled weight loss in the competitive and post-competitive periods.



An analysis of the frequency of recorded pre-pathological changes in the heart based on the electrocardiogram data of athletes resorting to an urgent decrease in body weight suggests that the training load in the competitive period should be regulated taking into account morphofunctional changes in the heart of athletes, taking into account its initial state. Hypertrophy of the left ventricle, revealed in the overwhelming number of examined wrestlers, requires ongoing ultrasound examination for the earliest detection of hypertrophic cardiomyopathy in them (Karpov et al., 2018; Zavalishina S.Yu. (2018b). Timely differentiation of pathological changes in the heart will allow timely removal of athletes from training with the appointment of rehabilitation measures, as well as make the necessary adjustments to their educational process, significantly reducing the risk of heart disease in these wrestlers (Zavalishina, 2018c; Zavalishina, 2018d).

Figure 3: The frequency of occurrence of functional disorders and electrocardiograms of fighters against the background of extreme weight loss in the competitive and post-competitive periods.



An analysis of the frequency of recorded pre-pathological changes in the heart based on the electrocardiogram data of athletes resorting to an urgent decrease in body weight suggests that the training load in the competitive period should be regulated taking into account morphofunctional changes in the heart of athletes, taking into account its initial state.The results obtained suggest that it is necessary for the coaching staff to exclude cases of extreme weight loss of an athlete, especially in light and medium weight categories. This will avoid the stress reactions of their body, especially in their cardiovascular system, which are always manifested in a change in the electrocardiogram indices (Zavalishina, 2018e; Zavalishina, 2018f).The result of restoring the reserve functions of the cardiovascular system in the post-competition period allows us to create a prognostic model for planning a competitive weight category, adequate physical activity in the annual cycle of training athletes without disrupting their adaptation processes and developing pathology (Zavalishina, 2018g; Zavalishina, 2018h).

CONCLUSION

Sinus arrhythmia, sinus bradyarrhythmia, early ventricular repolarization syndrome, I degree atrioventricular block, left ventricular hypertrophy, registered on the electrocardiogram, can be considered as a variant of a "sports heart", not being a pathology of the cardiovascular system. Changes in the electrocardiogram in the post-competitive period in the form of disturbances in the processes of myocardial repolarization, sinus arrhythmia and sinus tachycardia are manifestations of stress cardiomyopathy and a criterion for inadequate physical activity. This requires the removal of athletes from training sessions and metabolic therapy in order to restore the functional reserves of their cardiovascular system. Athletes who resorted to extreme weight loss during preparation for the competition recorded sinus tachycardia and sinus tachyarrhythmia, as well as cardiac

arrhythmias in the form of supraventricular extrasystole. These changes also require temporary suspension of athletes from stress and the appointment of rehabilitation measures.

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Enhancing Plant Growth by Chicken Feather Compost Obtained from Feather Degradation by *Streptomyces enissocaesilis*

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ABSTRACT

Nowadays, feathers are a major by-product of the poultry industry. Due to the increasing production of feathers from poultry industries, the untreated feathers could become pollutants. Feathers account for 5-7% of the total weight which is constituted of 90% keratin. Bioconversion is widely accepted as a low-cost and environmentally gentle process but limited by the availability of safe and highly efficient feather degrading bacteria. In this study, 15 actinomycete isolates were isolated, purified and screened for keratinase production using solid and broth media. Out of the 15 isolates, 9 recorded keratinase activities. The isolate AM1 was the most active one, thus it was selected for further studied. Using morphological, physiological and biochemical characters in addition to 16S rRNA, it was identified as *Streptomyces enissocaesilis* AM1with 95% similarity level to *S. enissocaesilis*. This isolate can efficiently degrade feathers. Keratinase enzymes from *Streptomyces enissocaesilis* AM1 showed optimal activity at pH 7 and 50°C. Mechanism of degradation includes, sulfitolysis, proteolysis, followed by deamination. In conclusion, *Streptomyces enissocaesilis* AM1 can grow on keratin as a carbon sourceand secret keratinase which degrade keratin tosmall peptide chains, amino acids, and minerals which can be used as organic fertilizer for enhancing plant growth.

KEY WORDS: FEATHER, DEGRADATION, STREPTOMYCES ENISSOCAESILIS, KERATINASE, PLANT COMPOST.

INTRODUCTION

Feather waste, generated in large quantities as a byproduct of commercial poultry processing, is nearly pure keratin protein (Moran et al., 1966). Keratin in its native state is not degradable by common proteolytic enzymes such as trypsin, pepsin and papain. However, keratin does not accumulate in nature and keratinolytic activity has been reported for many bacterial and fungal genera like *Bacillus* sp. (Aly et al, 2019) and *Streptomyces* (Fuhong et al., 2010, Tork and Aly, 2019), *Aspergillus* and *Ctenomyces* (Gupta et al., 2002). Importance enzymes were reported from bacteria (Aly et al., 2020, Bahamdain et al., 2020, Tork et al., 2020a, b).

Currently, feather waste is utilized on a limited basis as a dietary protein supplement for animal feed stuffs. A current value-added use for feathers is the conversion to feather meal, a digestible dietary protein for animal feed, using physical and chemical treatments. These methods can destroy certain amino acids and decrease protein quality and digestibility (Moritz and Latshaw, 2001). The nutritional inferiority and insolubility of native feather protein derive from the composition and

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molecular configuration of constituent amino acids that ensure the structural rigidity of feathers (Parry and North, 1998). Resistance to proteolytic enzymes has been attributed to the complex structure of keratin filaments. In addition, disulfide cross-links produce a compact three dimensional network, as a result of intermolecular disulfide bonds between rod domains and terminal domains of the constituent molecules (Parry and North, 1998).

The nutritional upgrading of feather meal through microbial or enzymatic treatment has been described. Feather meal fermented with *Streptomyces fradiae* and supplemented with methionine resulted in a growth rate of broilers comparable with those fed isolated soybean protein (Elmayergi and Smith, 1971). The crude keratinase enzyme increased the digestibility of commercial feather meal and could replace as much as 7% of the dietary protein for growing chicks (Odetallah et al., 2003). Keratinolytic microorganisms and their enzymes may be used to enhance the digestibility of feather keratin. They may have important applications in processing keratin-containing wastes from poultry and leather industries through the development of non-polluting methods (Onifade et al., 1998).

Generally, an increase in keratinolytic activity is associated with thermophilic organisms, which require high energy, inputs to achieve maximum growth and the decomposition of keratin wastes (Nam et al., 2002, Tork and Aly, 2019). Actinobacterial isolates can degrade raw feathers and therefore useful to develop efficient processes involving keratin substrates. In this study, we described the collection of feather dumping soil from several areas, isolation of Actinobacteria from feather dumping soil and selection of keratinolytic actinobacterial isolates by performing primary and secondary screening.

MATERIAL AND METHODS

Keratinolytic strains and culture medium: The chicken feather-degrading strain Streptomyces enissocaesilisAM1 was identified by screening. In this study, *Streptomyces enissocaesilis* AM1 cultured in a chicken feather medium (initial pH 8.0) comprising (g/L): chicken feathers 50, yeast extract 1.5, glucose 3.0, KH2PO4 0.7, K_2HPO_4 1.4, NaCl 0.5, and $MgSO_4$ 0.1.

Screening of keratinolytic actinobacteria: Screening of keratinolytic actinobacteria First, the keratinolytic activity of the isolated actinobacteria was determined in Milk agar medium (Riffel and Brandelli, 2006). The isolates that showed efficient keratinolytic activity were subjected to a second screening in modified basal liquid medium supplemented with raw chicken feather, $MgSO_4$, $7H_2O$ 0.2 g/l; K2HPO$_4$ 0.3 g/l; KH2PO$_4$ 0.4 g/l; $CaCl_2$ 0.22 g/l and Yeast extract 0.1 g/l were used to prepare the modified basal liquid medium (Aly et al., 2019).

Keratinase assay: Keratinase activity was determined by the modified method of Letourneau et al. (1989). Keratin azure (Sigma-Aldrich, USA) was used as the substrate. It was first frozen at – 10°C and then ground into fine powder by using Oscillating mil mm400 retch (Figure 3.1). Keratin azure powder (5mg) was suspended in 1 ml of 50 mM Tris-HCl buffer (pH 8.0). The reaction mixture contained 1 ml keratin azure suspension and 1 ml crude enzyme. The reactions were carried out at 50°C in a water bath for 30 min. After incubation, the reactions were stopped by adding 2 ml 0.4 M trichloroacetic acid (TCA) and followed by centrifuging at 3000×g for 5 min to remove the substrate. The supernatant was spectrophotometrically measured for the release of azo dye at 595 nm. One-unit (U) keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under given conditions.

Characterization of keratinolytic isolate AM1: Morphological and biochemical characterization of the keratinolytic isolate was carried out. The morphology of the spore-bearing hyphae with the entire spore chain, and the substrate and aerial mycelium of the strain were examined by light microscope. The isolate was compared and identified according to Bergey's Manual of Determinative Bacteriology. The second step is DNA sequence which was compared to the GenBank database at the National Center for Biotechnology Information (NCBI) using the BLAST program.

Enzyme purification: Solid ammonium sulphate (80% w/v) was used to the filtrate with gentle stirring at 4°C overnight. The mixture was then centrifuged at 8000 rpm for 30 minutes at 4°C. Both enzyme activity and protein content were determined in the precipitate. This purification step was carried out to remove the traces of ammonium sulphate. The resultant precipitate was dissolved in 5 mℓ 0.02 M tris-HCl buffer pH 8.5 and dialyzed overnight against 2 liters of the same buffer in a cellophane bag (Aly et al, 2020). The concentrated and dialyzed cell free supernatant became ready to be applied on further purification step. The dialyzed solution was concentrated under vacuum and applied to a column (30x1.5 cm) of Sephadex G100 column chromatography followed by diethylaminoethyl-cellulose (DEAE cellulose) and elution was with 1M NaCl in phosphate buffer at a flow rate of 80 ml/h and analyzed by UV spectrophotometer at 280 nm. The purity of the isolated protein was determined by the SDS-PAGE on 10% gel according to the method of Laemmli (1973). This method was used to determine the molecular weight of the purified keratinase enzyme. The molecular weight of enzyme was determined by standard protein markers (low molecular weight 14-60 kDa) with different molecular weights.

Effects of pH and temperature on keratinase activity: The effects of pH and temperature were assayed with keratinazure as substrate. Keratinase activity was studied in the pH range of 5•0–9•0. Optimum temperaturekeratinase activity was determined by varying the incubationtemperature between 20 and 80

°C. After growth period, the AM1 isolated keratinase activity were measured.

Molecular detection of Keratinase gene: In order to confirm the presence or absence of Keratinase gene inthe isolations strain by PCR technique for amplifying, the primer sequences were ordered and used. Gel electrophoresis was used to analyses the PCR yields.

Preparation of Feather Compost: For preparing soil Fertilizer from chicken feather, 20 g of sterile chicken feather were mixed in plastic bag with 1000 g sterile soil each which was pre-autoclaved at 121 °C for 15 min. Similarly, 20 g of non-sterile chicken feather was mixed with 1000 g of non-sterile soil. The preparations were then uniformly inoculated with 200 ml overnight culture suspension of isolated bacteria. Soil mixing was done aseptically, and each bag was labelled appropriately. A separate control without bacterial culture addition was prepared. The feathers were kept for degradation for 30 days, respectively. After 30 days degradation process, the treatments showing ≥ 95% feather degradation were selected for the physicochemical parameter analysis and for further pot study experiments.

Table 1. Keratinase production (U/ml) by the different actinomycete isolates grown in Mineral feather broth medium.

Actinomycete isolates	Enzyme Detection on solid medium	Enzyme Activity (A595)	Enzyme Activity U/ml	Actinomycete isolates	Enzyme Detection on solid medium	Enzyme Activity (A595)	Enzyme Activity U/ml
AM1	+++	0.656±0.038	0.494	AM6	+	0.045±0.133	0.032
AM2	++	0.117±0.144	0.073	AM7	+	0.047±0.002	0.039
AM3	++	0.178±0.019	0.159	AM8	++	0.451±0.224	0.370
AM4	++	0.151±0.023	0.118	AM9	+	0.064±0.009	0.055
AM5	++	0.371±0.05	0.221				

+++: high production, ++: moderate production, + Low: production

Figure 1: Keratinase production (U/ml) by the different actinomycete isolates grown in Mineral feather broth medium.

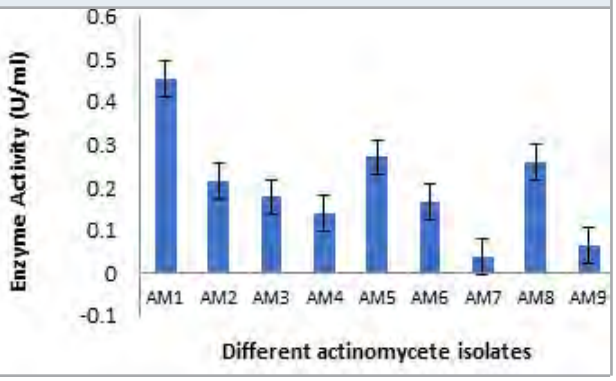


Figure 2: A) Secondary screening of keratinolytic actinobacteria on modified basal liquid medium. B) Primary screening.

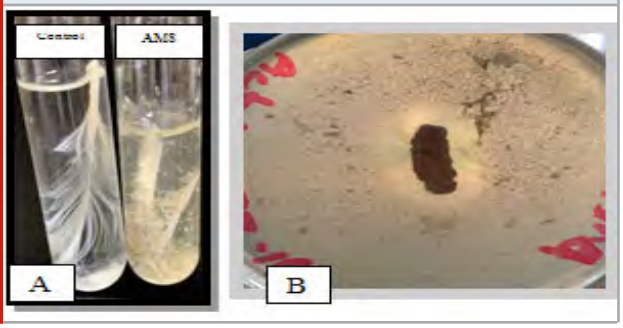


Figure 3: The selected actinobacterium AM1in A: Broth feather medium, B:on Starch agar c: under light microscope x 1000 after Gram staining.

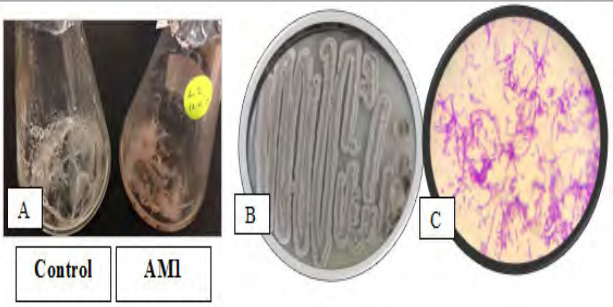
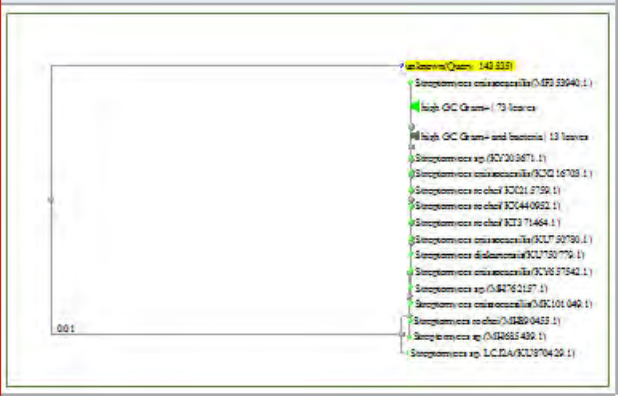


Figure 4: Phylogenetic tree of the isolate AM1 and most related genera.



RESULTS AND DISCUSSION

Isolation of Keratinase Producing Bacteria: A total of nine of the actinobacterial strains (AM 1-9) were isolated from the poultry waste and evaluated for their Keratinolytic activity on skim milk agar media and screened at raw feather broth medium for feather degrading property. Among the nine isolates the maximum keratinase enzyme production, observed at the 5th day incubation, was obtained byAM1 isolate as recorded in Table 1 and Figure 1. Out of the 9 isolates, isolate AM1 produced 0.456±0.038U/ml and it was the most active on solid and in broth medium. (Figure 2 A, B) showed the keratin degradation by the isolate AM1 in liquid and solid media. Growth profile of strain AM1 indicated that the selected strain efficiently utilized chicken feather as sole source of carbon and nitrogen. The strain AM1 isolated in the present study has an increased keratinolytic activity which is a desired potential characteristic feature. Thus, it was identified and characterized.

Identification of the Selected Bacterial strain: The keratin degrading actinobacterial isolate AM1 was Gram positive,filamentous bacterium, non-motile in nature and colony characteristics and morphology were examined (Figure 3). The biochemical testes of keratinolytic culture were also studied and the results were shown (Table 2).

Based on morphological characterization and 16S rDNA sequence analysis, AM1 strain of this study was identified as *Streptomyces enissocaesilis* and designated as *Streptomyces enissocaesilis* AM1 (NCBI GenBank Accession No. MF092720). The Phylogenetic tree of the isolate AM1 and most related genera was represented in Figure. 4.

Table 2. Some physiological and chemical characters of the selected actinomycete isolate AM1

Character	AM1	Character	AM1
Gram stain	+	keratinase	+
Catalase	+	Growth at 45°C	+
Oxidase	+	H2S production	+
Urease	+	Melanin production	+

The enzyme production by the selected isolate was carried out between temperature 25- 50°C and pH 5-9. It was found that the highest enzyme production by the selected bacterium was observed at 50°C and pH 7 (Figure 5).

Purification of keratinase: The *Streptomyces enissocaesilis* AM1 was cultivated for 5 days for all subsequent experiments. Purification of the keratinase was then undertaken. The crude enzyme, which was concentrated by centrifugation and precipitation with 80% saturation of ammonium sulfate. The precipitate was dialyzed and subjected to gel filtration on a Sephadex G-75 column. The elution profiles for keratinase and protein from the Sephadex G-75 column. Two protein peaks were obtained. The second peak shows the highest specific keratinase activity (243.2 U/mg of protein). The most active fractions (numbers 16-22) from the Sephadex G-75 column were pooled and further purified by DEAE-Sephadex A-50 column chromatography. Enzyme detection in the eluate revealed an active peak with high keratinase activity in the active fractions numbers 9-13. An overall recovery of 11.53-fold with a recovery of 32.36% and a specific activity of 316.15 U/mg protein were obtained (Data not shown). Analysis by SDS-PAGE revealed a single protein band. The molecular mass of keratinase of *Streptomyces* enissocaesilisAM1was estimated to be 66kDa by SDS-PAGE gel electrophoresis (Figuer 6 and 7).

Figure 5: Effect of Temperature and pH on keratinase production by the isolate AM1 in broth medium

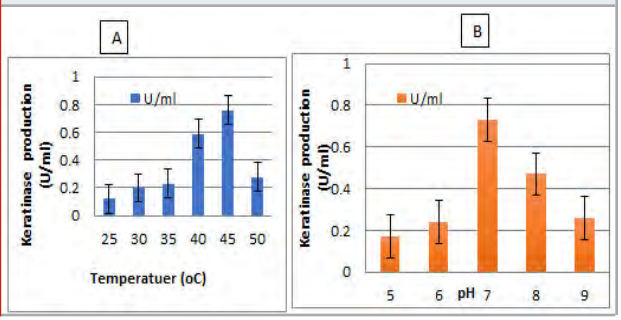


Figure 5: Purification of the Keratinase using two different columns chroma to graphy

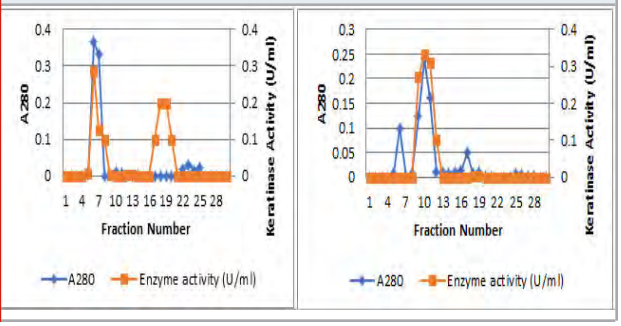


Figure 6: SDS-PAGE analysis of keratinase obtained from bacteria, Lane 1: purified keratinase by column chromatography, Lane 2: Marker.

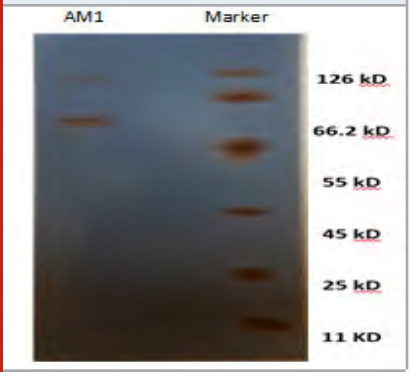


Figure 7: Effect of Temperature and pH on keratinase activity by the isolate AM1 Molecular detection of Keratinase gene in *Streptomyces enissocaesilis* AM1 by PCR.

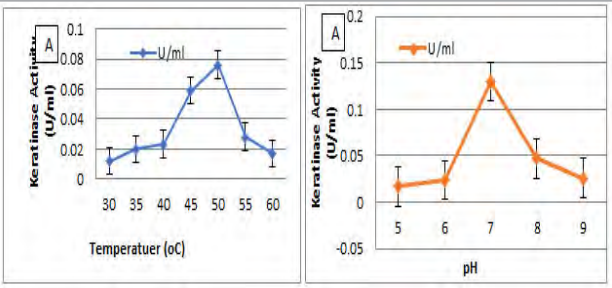


Figure 7: PCR amplification of Keratinase gene from *Streptomyces enissocaesilis* AM1 Lane 1:100bp ladder; Lane 2; 47bp partial Keratinase genes.

Effect of temperature and pH and on keratinase activity: The optimal temperature and pH for keratinase activity were determined (Figure 7). The optimal temperature for keratinase activity was determined by varying the reaction temperatures, between 30 ^{0}C and 60 ^{0}C, at pH 7. The optimal temperature was 50 ^{0}C for keratinase activity, but above 60 ^{0}C the activity sharply decreased, as shown in Figure7A. However, the enzyme was completely inactivated at more than 60 ^{0}C. The optimal pH for keratinase activity was 7 andthe enzyme activity declined rapidly at a pH higher than 8.0.

The gel electrophoresis image showed that Keratinase gene was detected in *Streptomyces enissocaesilis* AM1. Keratinase gene presence in *Streptomyces enissocaesilis* AM1was detected by PCR yield of Keratinase genes at 476bp (Figure 8).

Preparation of Feather Compost: After 30 days of degradation process, it was observed that there was no visible feather degradation in the control and cultureless treatment. It is indicative that during direct application of raw feather waste to soil, their degradation is augmented by the addition of microbial culture, else the process is hindered. The 10 g of feather treatment were completely degraded (Figure 8). The results indicated that for the effective degradation of keratin rich chicken feather, presence of specific *Streptomyces enissocaesilis* AM1 is required for enhancing degradation Composting of residual feather seems to require the presence of a co-substrate for composting and nitrogen conservation. Recently many works have been published on the biodegradation of animal wastes using specific microbial populations. Gushterova et al. (2005), Tiquia et al, (2005) obtained 50% carbon conversion when composting the wastes from the poultry industry with high nitrogen content indicating high biodegradability of protein of animal origin. The selected treatment soil sample which showed complete feather degradation were analyzed for physiochemical properties. An increase in N, P, K content was obtained with increase in feather compost percentage in soil.

Figure 8. A): The feather compost after 30 days of degradation. B) the feather compost after drying and prepared for growth plating.

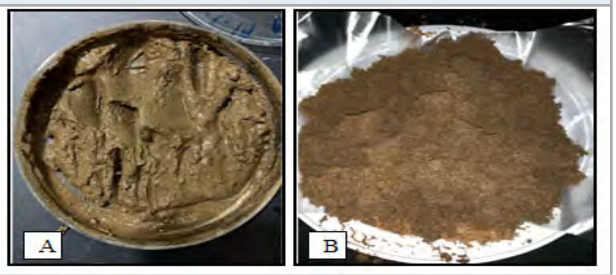
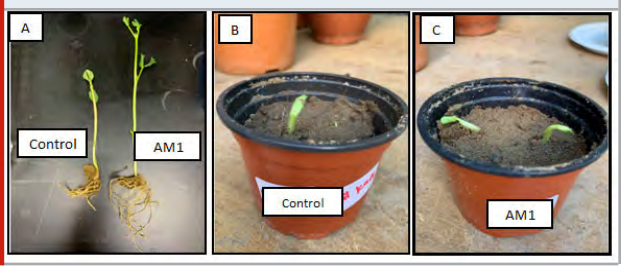


Figure 9: Plant growth under feather treatment of soil; A) broad bean growth, B) control plant, C) treated soil with feather compost.



Plant Growth Experiment: After 7 days of seed germination, it was observed that the plant growth in treatment sample exhibited high plant growthparameters when compared to control (Figure 9). The plant growth measurements namely shoot, root and leaf length were shown in Figure 9A.

Actinomycetes may have important role in processing keratin-containing wastes. Biodegradation of feathers

by keratinase from *Streptomyces* may provide viable alternative sources. Various species of Actinomycetes have been reported for feather degrading activity by keratinase (Singh et al., 2012). As it was reported that proteases specially keratinase were secreted by Genus Streptomyces and these enzymes presented activity at a wide range of pH (7.0 to 9.0) and temperature (30°C to 40°C). The added value of feather is its conversion by using physical and chemical treatments to dietary meal for animal feed or as a fertilizer for poor soil. These methods can destroy and decrease protein quality and digestibility of meal. Keratinase may be used to digest keratin. Ability of genus *Streptomyces* to degrade keratin into economically useful keratin product i.e. nitrogenous fertilizers, biodegradable films, glues and foils are well known. The enzymatic ability of the genus *Streptomyces* in large scale for decomposing feathers was not studied well while they were playing many important roles in carbon cycle in the environment.

All studies revealed the use of actinomycetes in degradation of keratin waste through is much safe and friendly for human and the environment than other commercial used methods. The aim of this study was to identify and isolate the keratinolytic actinomycetes from poultry farms wastes of Jeddah region. The most active isolates was identified using morphological and molecular methods as a spices belong to genus *Streptomyces*. Poultry feather degradation property of *Streptomyces enissocaesilis* AM1could be efficiently utilized in feather waste management. This study is useful in rapid removal of the recalcitrant feather content with the release of valuable by products acceptable in land use application. Microbial augmentation to compost at correct inoculum ratio can bring rapid and complete feather reduction to support increase in the quality of soil and growth of plants. The compost prepared from feather degradation along with bacterial strain could be successfully employed as an economic source of nitrogen fertilizers for plants. Addition of composts increases the nutritive value of soil. These feather compost characteristics thus increase the value of feather waste in agricultural field.

CONCLUSION

Keratinase enzymes from *Streptomyces* enissocaesilis AM1 showed optimal activity at pH 7 and 50°C. Mechanism of degradation includes, sulfitolysis, proteolysis, followed by deamination. In conclusion, *Streptomyces* enissocaesilis AM1 can grow on keratin as a carbon sourceand secret keratinase which degrade keratin tosmall peptide chains, amino acids, and minerals which can be used as organic fertilizer for enhancing plant growth.

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Seasonal Dynamics of Cattle Thelaziasis in Northern Trans-Ural Region and Pathogenetic Mechanisms of its Clinical Manifestations

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ABSTRACT

Thelaziasis of cattle is a helminthic disease caused by parasitizing of nematodes of a suborder of Spirurata, the Thelaziidae family. The primary purpose of this study is to elaborately analyze the seasonal dynamics of thelaziasis in cattle, terms of its invasive thelaziasis and the specification of the clinical implication of disease in the cattle in the Northern Trans-Ural region. The researches were conducted in 2002-2016, surveyed 27122 heads of cattle belonging to the agricultural enterprises and citizens. It can be concluded that under the conditions of the Northern Trans-Ural region, clinical signs of thelaziasis are shown from the first decade of May (EI of 3.39%). Besides, by the end of the month, this indicator increases more than twice (EI of 7.04%). Moreover, in the summers, the number of clinically sick thelaziasis animals are the highest. Clinical implication of thelaziasis is characterized by the development of the catarral exudative inflammation which is quickly changing on, in the beginning, is purulent - catarral, then there is a loss of transparency of cornea, fusion of tissues of eye and formation of erosion and ulcers on it. Due to the lack of treatment, there is a perforation of an iris of the eye or shape of a white or red cataract that may well result in the loss of sight and premature rejection of a sick animal.

KEY WORDS: CATTLE, THELAZIASIS, SEASONAL DYNAMICS, CLINICAL IMPLICATION.

INTRODUCTION

Considerable changes in the political climate around the world are reflected in an economic situation and our country. Sanctions and conditional isolation of Russia resulted in developing the different industries in Russia. The special attention is focused on the development of the agro-industrial complex due to which there is an import substitution of food products and implementation of the program of food security. Recently providing the population with fowl and pork reached absolute measures, there is not enough only beef meat (De León et al., 2020).

Cattle breeding - rather expensive and technologically complex branch of agriculture but to replace beef meat which contains full-fledged protein a large amount of iron at the same time rather dietary, today in our country it is not possible. Although the development of cattle breeding is under close attention and control of the state, it is not

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possible to provide the reason for that the population beef fully. During the summer period the most of cattle pasture on the remote pastures, mostly it is relevant for animals of the meat direction that spend on pastures about half a year (Kennedy and MacKinnon, 1994; Zubairova and Ataev, 2010; Bespalova and Grigorjeva 2015; Khedri et al., 2016; Bespalova et al., 2016; Chowdhury et al., 2018). Except for traumatic damages, the pasturable type of contents is dangerous to animals by an invasive parasite and continuous influence of insects and ticks (Stolbova et al., 2014). One of the widespread summer diseases in the Northern Trans-Ural region is thelaziasis (Glazunova et al., 2018; Glazunova and Glazunov, 2018; Luo et al., 2020).

Thelaziasis of cattle and the helminthic disease are mostly caused by parasitizing of nematodes of a suborder of Spirurata. (Sivajothi and Reddy, 2018; Munang'andu et al., 2011; Naem, 2007). Helminthes parasitize in channels of the lacrimal gland, the nasolacrimal channel, under the third century and in a conjunctival sac. Without treatment of this disease leads to a decrease in additional weights and milk yield of milk, and in the started cases to loss of sight and, as a result, to premature rejection (Safiullin, 1997). The infectiousness of cattle in some farms reaches 60-80% (Bespalova et al., 2016; Khedri et al., 2016; Otranto and Traversa, 2005). Cases of parasite invasion of specific parasites of cattle of Thelazia gulosa and Tskrjabiniare known at the person (Nath et al., 2008; Otranto and Dutto, 2008; Djungu et al., 2014; Krishnachary et al., 2014; Samardžiu et al., 2015; Bradbury et al., 2018).

Thelaziasis this seasonal disease as intermediate owners of thelazia are zoophilous flies. In the Northern Trans-Ural region, the pasture of animals is begun since the end of April of the beginning of May that coincides with the start of activity of zoophilous flies – intermediate owners of thelazia which become more active in the first from the second decade of April (dependence on meteoconditions) (Glazunova et al., 2018; Glazunova and Glazunov, 2018; Deak et al., 2020). Animals, having come to pastures, are infested in 14-28 days after the beginning of the attack on them of flies that is caused by the biology of the activator which metamorphosis passes an intermediate phase in an organism of these dipterous insects (Glazunova et al., 2018; Glazunova and Glazunov, 2018; Sivajothi and Reddy, 2018; Luo et al., 2020). The purpose of us researches was detailed studying of seasonal dynamics of thelaziasis at cattle and terms of invasive of cattle thelazia in the Northern Trans-Ural region as well as the specification of the clinical implication of disease at the cattle in the region.

METHODOLOGY

To study the distribution of thelaziasis among cattle, a clinical examination was performed involving animals belonging to 35 agricultural enterprises and the cattle from personal subsidiary farms from 2002 to 2016. During the period, 27122 heads of cattle were clinically examined. For reliability of the received results, only the cattle, which was not exposed to insecticidal processing, was examined. Some microscopic examination of washouts from a conjunctival cavity was conducted for confirmation of the diagnosis (De León et al., 2020). Data processing was carried out with the application of an indicator of the extensiveness of an invasion (EI). The obtained results were processed statistically (Prabhakar et al., 2015), taking into account average sizes, their mistakes and level of reliability (P) on Student on the computer with the use of the Microsoft Excel and Biostat program.

Thelaziasis: Thelazia parasite is a small white parasite that is about 1 to 2 cm in size. The skin of this parasite has prominent transverse lines that these protrusions or the same contrast of the cream cover cause lesions in the eye and its conjunctival tissue. As mentioned, this parasite causes infection in the eyes of ruminants and has been reported more in cattle. The parasite is found in the lacrimal duct of cattle and rarely in sheep, goats and buffaloes. This parasite has an indirect life cycle, i.e. it has an intermediate host, and without the presence of an intermediate host, the evolutionary process of the parasite does not take place. The parasite hosts of this parasite are species of flies that are swallowed by flies after 15 to 20 days of infancy when eating the first stage of Thelasia viable worms. This pathogenic baby, which is in the third stage, migrates to the oral appendages in the body of the fly and is placed in the conjunctival sac around the ruminant eye when feeding the fly. This parasite causes injuries and swelling of the conjunctival tissue and lacrimal duct by contrasting its covering, which causes tears to fall and in severe infections causes the cornea to become cloudy and sore.

RESULTS

As a result of the conducted researches, it is established that the cattle which is grazed on pasture or having a range during the day is exposed to an invasive thelaziasis. The extensiveness of thelaziasis invasion varied within 2.37-36.17% and averaged 10.79±0.98% (Glazunova et al., 2018). For clarification of seasonal dynamics of an invasivethelaziasis, we performed clinical examination of animals during all calendar year (table 1).

It is noticed that for the entire period of observation (from 2002 to 2016) the extensiveness of thelaziasis invasion before the beginning of a pasturable season (the first decade of May) and after its termination (the second decade of October) was minimum and made less than 1%. Changes in an epizootic situation happened practically right after an exit to a pasture. So, showing activity, zoophilous flies irritate a mucous membrane of eyes and lead to dacryagogue. At inspection of animals in May it is established that their quantity with clinical signs of thelaziasis increases by the end of the month. During this period, it is not possible to confirm the existence of thelaziasis invasion how larvae are not washed away from a conjunctival sac yet. So, if in the first decade of

May of animals in the herd with the clinic of thelaziasis there were 3.39%, then by the end of the month this indicator increases more than, twice and made 7.04%.

Allowed to confirm incidence thelaziasis further observation of animals when eventually they had bright clinical signs of disease and at the microscopy of washouts from a conjunctival sac found larvae of thelazia. In summer, the number of clinically sick thelaziasis animals is the highest. So, at the end of June, the extensiveness of an invasion was 12.36%. The number of sick animals increased and reached the maximum during the period from the third decade of July to the first decade of August with indicators of 15-83 and 15.36% respectively. In the second decade of August the number of the diseased still was at the high level – 12.23%. Then also by the end of September the number of animals with clinical signs of thelaziasis less than 5% systematically decreased (Figure 1).

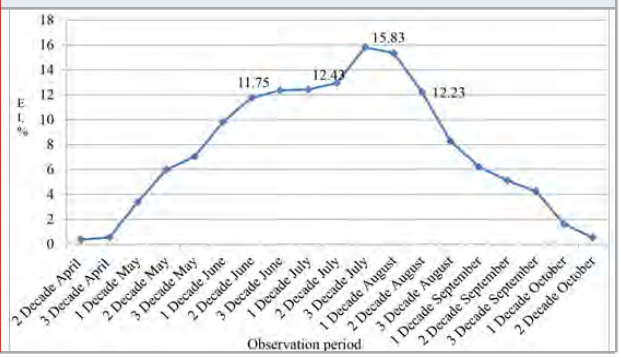
Table 1. Dynamics of manifestation of clinical signs of thelaziasis at cattle during the period from 2002 to 2016.

Research month	Quantity of the examined animals	Animals with clinical signs of thelaziasis	EI, %
		April	
2 decade	809	3	0,37
3 decade	1304	7	0,54
		May	
1 decade	118	4	3,39
2 decade	5085	304	5,98
3 decade	270	19	7,04
		June	
1 decade	2118	208	9,82
2 decade	2833	333	11,75
3 decade	1278	158	12,36
		July	
1 decade	2599	323	12,43
2 decade	3491	452	12,95
3 decade	4366	691	15,83
		August	
1 decade	1074	165	15,36
2 decade	564	69	12,23
3 decade	326	27	8,28
		September	
1 decade	306	19	6,21
2 decade	176	9	5,11
3 decade	94	4	4,26
October			
1 decade	124	2	1,61
2 decade	187	1	0,53
Total:	27122	2798	10,04±1,16

DISCUSSION

On the whole, the seasonal dynamics of cattle thelaziasis in Northern Trans-Ural region and pathogenetic mechanisms of its clinical Manifestations was fully analyzed. Thus, the high incidence of thelaziasis (higher than 10%) is fixed during the period from the second decade of June to the second decade of August. At the same time, it is necessary to consider that animals are infested much earlier than begin to show a disease clinically. It is noticed that during the winter period animals have no bright inflammatory reaction at thelaziasis, i.e. they have in a latent form and often are not exposed to preventive deworming therefore the cattle which is often infested by thelazia, coming to pasture, is an invasion source for other animals.

Figure 1: Seasonal dynamics of the clinical implication of thelaziasis at cattle (2002-2016)



So, allocations from eyes attract (physiological) arthropods, and they actively eat near an eye. During the food of a fly, the larva gets to the alimentary system of an insect, migrates, and there passes the development cycle. At repeated contact of the infested fly with an animal in 2-4 weeks' live larvae independently creep out of her proboscis and get into a conjunctival sac. The hit of larvae of thelazia in a conjunctival sac is followed by alternative processes in organs of sight, caused by the allergic and mechanical influence of a parasite (Luo et al., 2020). Clinically it is shown by hypostasis a century and photophobia (Figure 2).

Figure 2: The century, photophobia and dacryagogue swelled

The developing exudation at conjunctivitis defines the appearance of plentiful dacryagogue that in turn has protective value thanks to what inoculation of a parasite is possible. Besides, the development of local inflammation (conjunctivitis) has an immunosuppressant effect. It reduces the level of local nonspecific protection of tissues of the eye that contributes to the development of opportunistic microflora. The created vascular reaction is characterized by the formation of the catarral exudative inflammation characteristic of the conjunctiva, which is quickly changing on is purulent - catarral (Bespalova et al., 2016; De León et al., 2020).

At a progression of an inflammatory reaction, the cornea which being front transparent department of the external capsule of an eye globe is involved in the process, it is subject to the influence of all adverse environmental factors. Features of the building and metabolism of a cornea (anastomosing of the regional looped network of vessels of a cornea and an innervation, lack of own vessels, low level of exchange processes) explain its fast involvement in the pathological process and specifics of its current, characterized by loss of transparency of the cornea. Usually, this process develops promptly, and in 7-10 days after the first clinical signs of conjunctivitis inflammatory process passes to a cornea (Sivajothi and Reddy, 2018; Deak et al., 2020).

Development of larvae of thelazia in a conjunctival sac, after the third century and in the nasolacrimal channel supports secondary alteration, is followed by hyperplastic changes in cornea and transition of inflammation to an eye iris of the eye that is characterized by the development of iridocyclitis. Against the background of a progression of inflammatory reaction and its deepening in eye tissue as well as the reproduction of pyogenic microflora of response of an eye, the globe is various. In most cases at exudative keratoconjunctivitis and iridocyclitis under the influence of proteolytic enzymes of purulent exudate, there is a fusion of tissues of eye and formation of erosion and welcomes, and a perforation of an iris of the eye that comes to an end with the loss of sight (Chowdhury et al., 2018).

In the conclusion of the pathological process, during proliferation, on the cornea, the white or red cataract (see Figure 3 and 4) is formed. The white cataract is characterized by the fusion of a cornea and underlying tissues of an eye proteolytic enzymes and coagulation of proteins. The red cataract is formed at the expense of active vascularization of the damaged centre. In both cases, the injured eye loses the function. Much rarer, on the place of the inflammatory reaction, the new growth which growth is caused by an invasive of cattle thelazia during the summer period and development by long inflammatory response as well as the influence of solar radiation develops. Neoplastic changes most often are registered at cattle of Heleford breed that is promoted by a characteristic colour of the cattle. It is known that at the white-headed cattle of a tumour of eyes develop much more often than at animals with intensive pigmentation (Glazunova et al., 2018).

Considering pathogenesis of thelaziasis and its seasonal dynamics studying of the ecology of zoophilous flies – intermediate owners of thelazia is necessary for the development of a reasonable system of prevention of thelaziasis at cattle. Besides, considering pathogenetic bases of a current of thelaziasis for therapy, it is necessary to apply not only antiparasitic but also antibacterial, protective and immune performance-enhancing drugs which allow to adjust damages of a cornea and an iris of the eye of an eye and to prevent loss of sight as a result of turbidity of a cornea and formation on it of hems, perforations of an iris of the eye of an eye and development of new growths (see Figures 5 and 6).

Figure 3: A white cataract, as a result of the fusion of tissues of the eye

Figure 4: A red cataract, as a result of vascularization of the damaged centre

Figure 5: A hem on a cornea, after a recovery fromthelaziasis

Figure 6: A cornea perforation after a recovery fromthelaziasis

CONCLUSION

Thelaziasis is a worm disease stemmed from the Thelazia nematode, which is transmitted by flies of the infected family. In the current study, it was attempted to analyze the seasonal dynamics of thelaziasis at cattle and terms of invasive of cattle Thelazia in the Northern Trans-Ural region and the specification of the clinical implication of disease at the cattle in the area. It can be concluded that in summer, quantity clinically sick thelaziasis animals the highest. The maximum values fixed during the period from the third decade of July to the first decade of August (% EI 15.83 and 15.36 respectively). By the end of September, the number of animals with clinical signs of thelaziasis made less than 5%. During the stall period, the extensiveness of thelaziasis invasion was minimum and was less than 1%. The clinical implication of thelaziasis is characterized by the development of the catarral exudative inflammation, which is quickly changing on, in the beginning, is purulent - catarral. There is a loss of transparency of cornea, fusion of tissues of eye and formation of erosion and ulcers on it. In lack of treatment, there is a perforation of an iris of the eye or appearance of a white or red cataract that comes to an end with the loss of sight and premature rejection of a sick animal.

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Development of Resistance Against Tafenoquine in the Blood Induced Infection of *Plasmodium yoelii* and Its Possible Simultaneous Effect on Sporozoite Induced Tissue Stage

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ABSTRACT

Nearly about 50 % of the population is fighting the adversity of the malarial infections although there has been a huge researches as well as remedy development to bring down the incidence of malarial infections significantly. Moreover the emergence and transmission of resistance to antimalarial treatment continues to hamper malaria elimination efforts. The proportion of antimalarial use within the population and the presence of residual drug concentrations were identified to be the main predictors of the emergence and transmission of resistance. The present investigation focuses on whether the experimental resistance developed in the blood stage for Tafenoquine and Primaquine using serial passage on the basis of 2% delay time is also reflected in the tissue stage of the parasites life cycle as well. The experimental transmission of sporozoite induced infection of a rodent parasite *Plasmodium yoelii nigeriensis* through hamsters by serial cyclic passage using *Anopheles stephensi* as vector has been a potential test system for major causal prophylactic and anti-relapse antimalarial. In the undermentioned research drugs at a dose of 30mg/Kg were curative against the blood induced infection and became resistant to maximum tolerated dose of 40mg/Kg of Primaquine after XVII passages over a period of 240 days and for 90mg/Kg of Tafenoquine after XIX consecutive passages over a period of 218 days.The experimental transmission of sporozoite induced infection of a rodent parasite *Plasmodium yoelii nigeriensis* through hamsters using *Anopheles stephensias* vector has been a potential test system for major causal prophylactic and anti-relapseantimalarial. This test system will help evaluate novel strategies as alternative to the frequent extensive use of safer and potential drugs like Tafenoquine.

KEY WORDS: MALARIA, P. YOELII NIGERIENSIS, TAFENOQUINE.

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INTRODUCTION

The investigations throughout the world has found out that about half of the world's populations are vulnerable to the risk of Malaria. There have been huge efforts made to bring down the incidence of malaria significantly. The capability of malaria causing microorganisms to emerge, transmit and show multidrug resistance has been a hurdle in the treatment of the malarial infections.Moreover researches are on board, recently an 8 aminoquinoline,

Tafenoquine (WR238605), jointly developed by Walter Reed Army Institute of Research and Glaxosmithcline pharmaceuticals has shown its efficacy as 10 times more potent and safer with better bioavailability as blood and tissue schizontocidal compared to Primaquine (Geoffrey et al., 2007) Our present investigation focuses on whether the experimental resistance developed in the blood stage for tafenoquine and Primaquine using serial passage on the basis of 2% delay time (Walsh et al., 2009) is also reflected in the tissue stage of the parasite's life cycle as well which will also give insight into the mode of action of the drug at two different stages of parasite's life cycle (Peters et al., 1993Yehenewet al., 2016).

Any chemo-prophylactic drug, taken infrequently to improve compliance, should be well tolerated and highly effective against all stages of malaria. In contrast to the drugs active only against blood stages Primaquine has been the mainstay therapy for several years for clearing parasites from the liver (Peters et al., 1993). Primaquine has a low therapeutic index with serious side effects like methaemoglobin formation in G6PD deficient individuals (Walsh et al., 2009). In view of the avoidance of certain side effects a far safer prophylactic as well as blood schizontocidal 8 aminoquinoline WR238605 (Tafenoquine) with better bioavailability and longer half-life, has been synthesized (World Health organization, 2014; 2014; 2015 Geoffrey and Bryan 2017). However the efficacy of newest and safest drugs is challenged by ever increasing emergence of resistance (Ponsa et al., 2003; Shanks et al., 2001). Till date, emergence of resistance has been described for all the available drugs (Looareesuwanet al., 1999).The aim of present study is to investigate that the extensive use of the drug as blood schizontocidal might lead to a rapid emergence of resistance for the drug which can also hamper its efficacy as transmission blocker by being reflected at tissue stage as well. If it would so, the mode of action of the drug will be the same at both of the parasitic stages.

MATERIAL AND METHODS

Blood and sporozoite induced infections with rodent malaria parasite *P.yoelii nigeriensis* (strain N 67) in swiss mice maintained at this institute, have been used in the present study. Outbred Swiss mice of either sex weighing 22-25 g were procured from the animal facilities at the institute and maintained on commercial pellet diet and water ad libitum under standard housing conditions. Ethical guidelines on handling and use of experimental animals were followed during the conduct of the study.

Drugs: Doses were calculated as base mg /Kg.Pure samples of the drugs tafenoquine were procured from Walter Reed Army Institute of Research, Washington DC, U.S.A. were used for the evaluation.

Drug preparation: Suspension of Primaquine was prepared by simply dissolving it's disulphate salt in the water.For Tafenoquine, a paste prepared with 2-3 drops of tween 80 was suspended in water to make a suspension.

Drug administration: Drugs were administered orally through a modified round ended 18 gauge needle in 0.1-0.5 ml volume.

2% Delay time: The immune system of a vertebrate host is enhanced when the parasitaemia reaches beyond 2%. For determining the 2 % delay time, the drug is administered for single day or 4 days from 0-3 days and the time in attaining 2% for drug treated as well as control group is monitored (Merkliet al., 1976).The 2% delay time was calculated by subtracting the time for control group to reach 2% parasitaemia from the time in drug treated animals in 2% parasitaemia. Higher delay time is indicative of the drug's capacity to suppress the growth of the parasite.

Determination of curative dose level and the sequential selection of resistance for the drugs: The evaluation of blood schizontocidal activity in rodent model, mice were inoculated intraperitoneally with $1x10^6$ *P.yoelii nigeriensis* and treated with the drugs. The dose of 10mg/kg of Tafenoquine was on day 6,7 and 8 during 1st passage and a total of 30 mg/kg was administered followed by an increased dose of 50 mg/kg in 2nd passage shown in the table 3 and so on. Giemsa stained blood smears from the experimental animals were microscopically observed upto day 28 post inoculation. The animals fail to develop infection till day 28 were considered as cured. The parasitaemia levels from animals that developed patent infection were sequentially recorded to determine the time to patency in every serial passage. The two criteria for the assessment of development of resistance were: The gradual decline in sensitivity with serial passages showing early patency is indicative of development of resistance and the patency established before 28 days after the treatment with curative dose of the drug.

Cyclic transmission of the parasite: usually 3-5 days old *Anopheles stephensi* mosquitoes reared in the insectorium were allowed to engorge blood from an infected hamster carrying appropriate gametocytes numbers. The infected hamster was restrained to allow undisturbed mosquito feeding by injecting 50 mg/Kg of thiopentone which immobilizes the animal for 30-40 min.

Causal prophylactic activity: The treatment drug was administered in three doses on day -1,0, ±1 and animals were challenged intravenously with $1x10^5$ *P.yoelii nigeriensis* sporozoites on day 0.Blood smears were observed from day 3 to day 28 to record the day of patency (Puri, 2000).

Procedure for determining the mosquito infectivity:

- Ookinete determination by examining the partially digested blood meal within 16-20 hrs after blood meal recovered from the lumen of the midgut.
- Oocyst numbers monitored between day 4-7 after blood meal by dissection of midguts of infected mosquitoes.
- Sporozoite determinations made by examining the salivary glands on day 10-12 post blood meal.A minimum of 20 mosquitoes for each batch were

dissected and midgut infections with oocysts were quantified with regard to following parameters: percentage of mosquitoes positive for oocyst and mean number of oocysts / positive mosquitoes.

Dissections for salivary gland sporozoites: For *P.yoelii nigeriensis* transmission. The salivary gland dissections are usually carried out between 10-14 days post infective blood meal.

Harvesting and preparation of sporozoite inoculum: The mosquito thoraces ground and centrifuged in 1:3 serum saline mixture contains sporozoite.

Sporozoite inoculation to vertebrate host: A volume of 0.2 ml containing 1X 10^4 of the sporozoite suspension was inoculated intravenously using a 26 gauge needle and animals were observed from day 3 onwards to monitor the infectivity.

RESULTS AND DISCUSSION

Blood schizontocidal response of Primaquine and Tafenoquine was evaluated at three dose levels in 10 mice each after the 4 days regimen. The results (table 1) showed that animals treated at 30mg/Kg for both of the drugs (Primaquine and tafenoquine) did not develop patent infection till day 28 and hence were cured. Treatment at lower doses showed patency.

WR 238,605, a novel 3-phenoxy-substituted 8-aminoquinoline, possesses causal prophylactic, blood schizontocidal and gametocytocidal activity against rodent malaria parasites (Sinha et al., 2014). It has an established fact that this 8 aminoquinoline drug has shown its superiority in terms of efficacy as well as safety over primaquine against multidrug resistant lines of *P.yoelii* and *P.berghei* (Peters et al., 1993). In combination with chloroquine (CQ), WR 238,605 display a synergistic or 'resistance-reversing' action against CQ-resistant *P. yoelii* NS parasites (Llanos et al., 2014). In view of the present scenario with escalating resistance to available drugs and their safety issues Tafenoquine is a good candidate compound for clinical trials against multiresistant *P.falciparum* strain as well as a causal prophylactic and ant relapsing agent against *Plasmodium vivax* infections.

Table 1. Blood schizontocidal response of Tafenoquine against *P.yoelii nigeriensis* -67 in Swiss mice based on delay time in attaining 2% parasitaemia

Drug	Dose mg/Kg	No.of mice	Time to reach 2% parasitaemia	2% delay time relative to control group	Mean ±SE	Cure Rate%
	30	6	All protected			100
Tafenoquine	10	6	6,9,9,9,10,11	3.6,3.6,3.6,6.6,7.6,8.6	5.6 ±0.58	0
(WR238605)	5	6	3.8, 4,5,6.1,7,7	1.4,1.6,2.6,3.7,4.6,4.6	3.1 ± 0.58	0
Control		4	1.7,2,2.9,3 Mean ±SE2.4±0.39			

Table 2. Blood schizontocidal response of Primaquine against *P.yoelii nigeriensis* -67 in Swiss mice based on delay time in attaining 2% parasitaemia.

Drug	Dose mg/Kg	No.of mice	Time to reach 2% parasitaemia	2% delay time relative to control group	Mean ±SE	Cure Rate%
Primaquine	30	5	All protected			100
	10	5	6.7,7.3,7.5,7.6,7.9	4.0,4.6,4.8,4.9,5.2	4.7 ±0.19	0
	5	5	3.8, 5.02,5.4,5.5	1.1,2.3,2.7,2.8,3.1		
Control		4	1.7,2.6,2.9,3.6	Mean ±SE 2.7 ±0.39		

In our study also It has shown its blood schizontocidal as well as causal prophylactic efficacy against Chloroquine resistant *P.yoelii nigeriensis* (table1 and 7).Owing to the situation our study is aimed to investigate whether the frequent clinical use of this antimalarial would lead to development of resistance at blood induced stages and if so, would it be reflected at tissue stage as well. In our experimental study we tried to develop resistance for the drugs at blood stage which did not reflect at tissue stage of the parasite as the causal prophylactic activity assessed remained the same as before and after the development of resistance (table 8). The outcome of the study reveals that the site of action of the drugs are different against both the blood and the tissue induced infections as it were the same the resistance developed at blood stage would have reflected at tissue stage at the same time

with the altered causal prophylactic activity after the development of resistance.

No alteration in causal prophylactic activity is indicative of the different sites of targets for the drug. WR 238,605, a novel 3-phenoxy-substituted 8-aminoquinoline, possesses causal prophylactic, blood schizontocidal and gametocytocidal activity against rodent malaria parasites (Yehenew et al., 2016). It has an established fact that this 8 aminoquinoline drug has shown its superiority in terms of efficacy as well as safety over primaquine against multidrug resistant lines of *P.yoelii* and *P.berghei* (Yehenew et al., 2016). In combination with chloroquine (CQ), WR 238,605 display a synergistic or 'resistance-reversing' action against CQ-resistant *P. yoelii* NS parasites (Llanos et al., 2014). In view of the present scenario with escalating resistance to available drugs and their safety issues Tafenoquine is a good candidate compound for clinical trials against multiresistant *P.falciparum* strain as well as a causal prophylactic and antirelapsing agent against *Plasmodium vivax* infections. In our study also it has shown its blood schizontocidal as well as causal prophylactic efficacy against chloroquine resistant *P.yoelii nigeriensis* (table 1 and 2).

Table 3. Sequential development of resistance to Tafenoquine (WR238605) in a strain of *P.yoelii* (N-67) in Swiss mice

Serial passage no.	Duration of passage	Drug administration On day	Drug dose on respective days mg/Kg	Total dose administered mg/Kg
I	0-10	3,5,7	10,10,10	30
II	10-22	3,5,9,10,11	10,10,10,10,10	50
III	23-36	3,4,5,6	10,20,20,20	70
IV	36-54	7,8,10,12,14,15,16	10,10,10,10,10,10,10	70
V	54-68	6,7,8,9	10,10,10,10	40
VI	69-79	3,4,6,7	10,10,10,20	50
VII	80-90	0-3	30,30,30,30	120
VIII	91-100	0-3	30,30,30,30	120
IX	100-105	6	30	30
X	106-112	0-3	45,45,45,45	180
XI	112-120	0-3	45,45,45,45	180
XII	171-180	0-3	60,60,60,60	240
XVII	181-195	0-3	60,60,60,60	240
XIX	206-218	0-3	90,90,90,90	360
XX	219-230	0-3	90,90,90,90	360

Owing to the situation our study is aimed to investigate whether the frequent clinical use of this antimalarial would lead to development of resistance at blood induced stages and if so, would it be reflected at tissue stage as well. A Primaquine resistant strain of *P.yoelii* was selected after sequential exposure to drug for 16 sequential passages over a period of 218 days. During first passage, a total dose of 30mg/kg was administered. Dose of 10mg/Kg was administered on day 6, 7 and 8. During second passage the dose of 10mg/Kg was continued but total dose was increased to 50mg/Kg. The schedule and dose of drug administration upto XX passage is presented in table (6). The induction of resistance to Primaquine was observed by standard 4 day test initially by the 12 th serial passage table(7). During the 15 th passage also 3 out of 5 mice showed decline in Primaquine sensitivity. Gradual many fold resistance was observed with increased duration of exposure upto XX passages and the strain became resistant to maximum tolerated dose of the drug. Cyclical transmission of the parasite was carried out with varying grades of resistant parasitic line.

It was found that only low to medium level of resistance developed through many passages was able to get transmitted and to produce sporozoite in the mosquito vector. Likewise Primaquine, Resistance to Tafenoquine was also achieved through serial passages and cyclical transmission was done intermittently with various levels of resistant lines and similar results were obtained showing only low to medium level resistant parasite being capable of producing sporozoites (Alanoet al., 1995; Sinha et al., 2014). The most successful transmission of the parasite to mosquitoes was achieved just after XIII th passage for Primaquine and VIII th passage for Tafenoquine lines. A twofold resistance for Tafenoquine against blood induced infection of *P.yoelii nigeriensis* was developed and sensitivity towards curative dose (30 mg/Kg in a 4 day regimen) was evaluated.

A total of XV serial blood passages within a period of 170 days resulted in the development of two fold resistance in the parasite for Tafenoquine. The parasite load was also kept free of the drug pressure intermittently. The stability of resistance after cryopreservation (123 days)

and drug free maintenance (150days) was evaluated and found to be constant. The drug was evaluated for its blood schizontocidal as well as for its causal prophylactic activity before and after the development of resistance.

Table 4. Evaluation of sensitivity to WR238605during selection of WR238605 (tafenoquine) resistant strain of *P.yoelli* in swiss mice.

Serial passage no.	Treatment Duration in days	No. of mice	Dose mg/Kg	Mice showing nill parasitaemia						Day of patency
				4	7	10	14	21	28	
VII	0-3	5	30	5	5	1	0	0	0	8,8,9,9,12
		5	45	5	5	5	5	5	5	-
		5	60	5	5	5	5	5	5	-
VIII	0-3	5	30	5	5	1	0	0	0	9,9,10,10,12
XII	0-3	5	30	5	1	0	0	0	0	5,5,6,6,9
XIII	0-3	5	45	5	2	1	0	0	1	5,6,6,8,11
XIV	0-3	5	45	5	2	0	0	0	0	5,6,6,8,10
		5	60	5	5	5	5	5	5	-
XV	0-3	5	45	4	0	0	0	0	0	5,5,6,6,8
		5	60	5	4	0	0	0	0	7,8,8,8,10
XVI	0-3	5	60	2	1	0	0	0	0	4,4,5,6,9
XVII	0-3	5	60	5	5	0	0	0	0	8,8,9,10,13
XIX	0-3	5	90	1	0	0	0	0	0	4,4,4,4,6
XX	0-3	5	90	5	2	0	0	0	0	5,6,6,8,9

Table 5. Sequential development of resistance to Primaquine in a strain of *P.yoelii* (N-67) in Swiss mice

Serial passage no.	Duration of passage	Drug administration On day	Drug dose on respective days mg/Kg	Total dose administered mg/Kg
I	0-11	6,7,8	10,10,10	30
II	11-30	5,6,9,10,17	10,10,10,10,10	50
III	31-41	-	-	
IV	42-53	5	10	10
V	54-62	3	10	10
VI	63-74	3,10	10,10	20
VII	77-89	4,6,7,8,	10,10,10,10	40
VIII	90-112	6,8,9,10,11,12	10,10,10,10,10,10	60
IX	113-126	4,5,9,10,13	10,20,10,10,10,10	70
X	127-139	3,4,8,9,	10,10,10,10	40
XI	140-151	5,6,9,10	10,10,10,20	50
XII	152-164	0-3	30,30,30,30	120
XIII	165-173	3,4,5,7,8	30,30,30,30,30	150
XIV	174-185	2,6,7	30,30,30	90
XV	219-228	0-3	30,30,30,30	120
XVI	219-228	0-3	30,30,30,30	120
XVII	229-240	0-3	40,40,40,40	160
XIX	249-257	0-3	40,40,40,40	160

Table 6. Sensitivity test during selection of Primaquine Resistant strain of *P.yoeliiin* Swiss mice.

Serial passage no.	No.of mice	Treatment Duration in days	Dose mg/Kg	Mice showing nill parasitaemia on day						Day of patency
				4	7	10	14	21	28	
XII	5	0-3	30	5	5	2	0	0	0	8,10,10,11, 11
XV	5	0-3	30	5	5	5	2	2	2	11,11,12,12,13
XVI	5	0-3	30	5	2	0	0	0	0	7,7,7,8,9
XVII	5	0-3	30	5	1	0	0	0	0	6,6,7,7,8
XVIII	5	0-3	30	5	1	0	0	0	0	6,7,7,7,9
XIX	5	0-3	40	5	0	0	0	0	0	5,5,6,6,7
XX	5	0-3	40	5	0	0	0	0	0	5,6,6,6,6

Table 7 Causal Prophylactic activity: Primaquine and Tafenoquine

Drug/dose A) Primaquine	Treatment days	No.of animals	Day of patency	Mean value ± SE	No. of mice cured	Cure Rate%
40 mg/Kg	-1,0, ±1	5	All protected	0	6/6	
40 mg/Kg	0 day	6	All protected	0	6/6	100
30 mg/Kg	-1,0, ±1	6	5,6,6,6,7 -ve	5 ± 0.94	1/6	100
20 mg/Kg	-1,0, ±1	5	5,5,6,6,7	5.8 ±0.54	0/6	16.6
Control		6	5,5,5,5,5	5.0 ±00		0
B) Tafenoquine			All protected			
40 mg/Kg	-1,0, ±1	6	All protected	0	6/6	100
20 mg/Kg	0 day	5	All protected	0	6/6	100
10 mg/Kg	-1,0, ±1	5	11,11,11,12,12	0	6/6	100
40 mg/Kg	-1,0, ±1	5	All protected	11.4 ±0.21	0/6	0
10 mg/Kg		5	4,4,6,6,7	0	6/6	100
05 mg/Kg		5		5.4 ± 0.53	0/6	0
Control		5	4,5,5,6,6	5.2 ± 0.33		

The causal prophylactic dose of the drug (10 mg/Kg in -1, 0, ±1) remained unaltered even after the development of resistance in a blood induced infection of Chloroquine resistant *Plasmodium yoelli*. (Vuonget al., 2015). The unaltered causal prophylactic activity of the drug at tissue stage is indicative of the two different mode of actions of the drugs at blood and tissue stage of infections. Previous investigations on pharmacodynamic and antimalarial activity reveal that degradation of hemoglobin by malarial parasite generates heme which is detrimental to the parasite and detoxified through its conversion into insoluble pigment, hemozoin by the parasite itself as a defence mechanismas also discussed by (Vennerstromet al., 1999).

Tafenoquine accumulates within the food vacuoles and inhibits the detoxification of heme to hemozoin by the malarial parasite. It does so by inhibiting hematin polymerization by binding to its -oxo dimer, thus inhibiting the formation of hemozoin, which consists of cyclic heme dimers arranged in an ordered crystalline structure through intermolecular hydrogen bonding. Tafenoquine, via its hydroxylated metabolites, stimulates the hexose monophosphate shunt, increases methemoglobin production and decreases glutathione levels in the cells. The pro-oxidant properties of its metabolites correlate with its exoerythrocytic schizontocidal action and also contribute to its erythrocytic (Marcsisinet al., 2014) schizontocidal action.

Like PQ (Vuonget al., 2015) cytochrome P450 is supposed to be held responsible for the activation of TQ as it is metabolized by the enzyme, CYP2D6a (a liver microsomal enzyme) (Pybuset al., 2013) as was demonstrated by the CYP2D6 knockout mice lacking anti-malarial activity of TQ, This work could also be discussed with the works of Pradines and Brueckner (Pradines et al., 2006; Brueckneret al., 1998). This report from laboratory animals shows

Table 8. Causal Prophylactic activity of Primaquine and Tafenoquine after development of resistance

Drug/dose A) Primaquine	Treatment days	No.of animals	Day of patency	Mean value ± SE	No. Of mice cured	Cure Rate%
40 mg/Kg	0 day	6	All protected	0	6/6	100
30 mg/Kg	-1,0, ±1	6	5,6,7,6, 5	6 .0 ± 0.33	0/6	0
Control		6	5,6,5,6,5	5.4 ±00		
B) Tafenoquine						
	-1,0, ±1	5	All protected	0	6/6	100
10 mg/Kg	0 day	5	10,10,11,12,12	0	6/6	0
40 mg/Kg	±1 day	5	All protected	11.0 ±0.40	6/6	100
10 mg/Kg	-1,0, ±1	5	4,4,6,6,7	5.4 ± 0.53	0/6	0
05 mg/Kg						
Control		5	4,5,5,6,6	5.2 ± 0.32		

Note: With the sequential development of resistance, for Tafenoquine at 30, 45, 60 and 90 mg/kg and for Primaquine at 30 and 40 mg/Kg, the strain were tested intermittently for the gametocytes count and it's cyclical transmission, preservation being carried out in liquid nitrogen. The results obtained are shown in the table below. Preservation had no effect on cyclical transmission.

Table 9. cyclical transmission of the parasite at different resistance level for Primaquine and Tafenoquine resistant strain

Primaquine	Strain resistant to drug when given in 3 days regimen (0-3days)	Cyclical transmission of the parasite
Tafenoquine resistant strain	30 mg/Kg	± ± ± ±
	40mg/Kg	± ± ±
	30mg/Kg	± ± ± ±
	45mg/Kg	± ± ± ±
	60mg/Kg	± ±
	90mg/Kg	---

Note: In case of Primaquine resistant line only till XVIII passage, the cyclic transmission was satisfactory and went down further gradually whereas in case of Tafenoquine resistant line, the transmission slowed down after XII th passage. The strains were preserved at every level of resistance developed and tested for cyclical transmission and causal prophylactic activity.

the association between CYP2D6 metabolism and TQ pharmacokinetics. The follow up of the literature till date coincide with our findings and suggestive of the two different location of tafenoquine targets with two different tissue level organizations.

CONCLUSION

The study targeted the investigation on extensive usage of drugs as blood schizontocidal that has lead to the emergence in resistance of drugs which has hampered efficacies of treatment by being transmission blocker in tissues as well. The drug was evaluated for its blood schizontocidal as well as for its causal prophylactic activity before and after the development of resistance.In the above mentioned research drugs at a dose of 30mg/Kg were curative against the blood induced infection and became resistant to maximum tolerated dose of 40mg/Kg of Primaquine after XVII passages over a period of 240 days and for 90mg/Kg of Tafenoquine after XIX consecutive passages over a period of 218 days.The experimental transmission of sporozoite induced infection of a rodent parasite *Plasmodium yoelii nigeriensis* through hamsters using *Anopheles stephensias* vector has been a potential test system for major causal prophylactic and anti-relapseanti-malarial. This test system will help evaluate novel strategies as alternative to the frequent extensive use of safer and potential drugs like Tafenoquine.

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Contmann: A Tool to Calculate Contact Distances Between Amino Acid and Mannose Using Protein Data Bank File at Distance Cutoff

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ABSTRACT

Protein function depends on interaction with their ligands and mannose is one of the important ligands for understanding glycoprotein, so it is often required to calculate the binding site residues in protein at different distance threshold from PDB file. To study particular protein chain and its interaction with mannose in complex form, researchers have to parse the output of different available tools or databases for binding-site residues. Here we have developed a tool for calculating amino acid contact distances in proteins at different distance threshold using PDB file. ContMann can quickly find all binding-site residues in the protein by calculating distances from its coordinate present in pdb file by selecting the different distance threshold, Additionally, it can also generate atomic details of contacts including distances of binding-site residue. ContMann tool is available at: http://procarb.org/procarbdb/cfind-contact2.html

KEY WORDS: PROTEIN FUNCTION DEPENDS, CONTACTS INCLUDING DISTANCES..

INTRODUCTION

The proteins function depends on interaction with their ligands, among ligands Mannose is very important (Turner MW, 2003, Ng KK, 2002). Therefore, identifying amino acid contacts is important for understanding the glycoproteins. In order to understand the interactions calculating the amino acid contacts at different distance thresholds required (Kenneth, 2002). Binding site residues of proteins can also be identified from databases (Desaphy 2015), visualization tools (Jendele 2019), or many other web servers developed earlier (Jendele 2019, Angles 2020), but this becomes overwhelmingly imposing when a large set of proteins have to be analysed.

With the help of this tool, user can get the binding residue by after uploading PDB file. Additionally, it can also generate atomic details of contacts including distances of binding-site residue from PDB structures. Protein Data Bank (PDB) is repository of for 3D structures of biological macromolecules which has coordinates of its atoms (Kayikci 2018; Berman 2003), using these coordinates of two atoms, this tool can compute the distance between them.

A residue is defined as a binding residue if the distance between atoms of the interacting partner is less than a certain distance cut off (Eyal 2001). Upon uploading the protein mannose 3D-structure file of interest and option selected for distance threshold by user, ContMann searches the PDB file for the protein chains, Ligand chain of interest and the number of protein models (if multi model protein). If more than one model is present,

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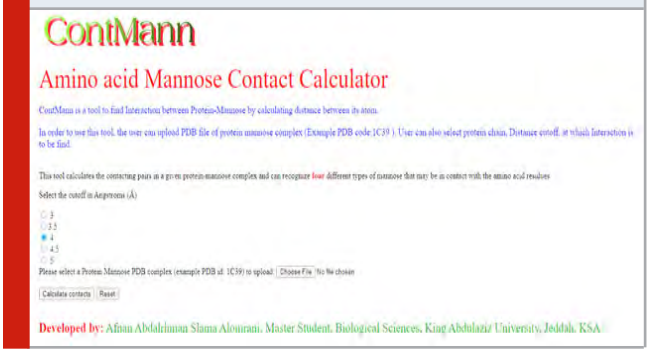
ContMann gives option to select and parse the desired model present in the uploaded Protein Data Bank (PDB) file. Then ContMann calculates the distance between selected protein chain residue atoms and interacting partner atoms, and when this distance falls below or equal to the selected distance threshold, this residue is considered as binding residue. The overall description is illustrated at home page (Figure 1).

Web Interface: Web interface of current version of ContMann developed using HTML, JavaScript and CGI-PERL scripting language. It has home page where description about this tool mentioned, uploading pdb file option and submit button provided, here user can also select distance threshold in angstrom (Figure-1)

Figure 1: Screen shot of ContMann Home page. From this home page user can upload PDB file and select distance threshold.



Program input: The input to the ContMann is a protein 3D coordinate PDB file, modeled protein or a docked complex file. The user can select provided distance threshold also (figure 1).

Figure 2: Screen shot of output page. Here user can find the interaction residues with its atom, distances in angstrom and summary of Amino acid contact with mannose.

Program output: At the top of the program's result page (figure 2), the uploaded file name and distance threshold selected for the calculations are displayed. The calculated distance between the two atoms, its residue, protein chain and the interacting atoms is displayed in a tabular form (figure2). The page summary section has total number of contacts at the end of page. First five column has distance, residue, position, atom and chain of protein and last 3 column has ligand three letter code, atom and chain of ligand. At the end of output summary of binding residue also mentioned.

Proteins are complex and have different conformations, that are often related to the protein function (Karplus 2005). Mannose binding proteins act as receptors and take part in innate immunity, human mannose-binding protein also has role in first line host defense (Ezekowitz 2003; Turner 2003). Identification and analysis of binding sites of protein with mannose are important and needful.

CONCLUSION

The developed tool will be useful for the identification and analysis of binding sites residue of protein from 3D-structure PDB file of protein mannose complex at different distance threshold. Although, in current version only one pdb file can be uploaded but it will be upgraded for batch file, so user can upload list of files.

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Application of 16 rRNA Gene of V3-V4 Region for Meta Barcoding of Bacterial Community in High Density Population of Eastern India

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ABSTRACT

The treatment of wastewater technologies depends on a composition of physio- chemical as well as biological factors. Bacteria present in wastewater treatment plants, which may play an essential role for the degradation, mitigation and removal of organic waste and xenobiotic pollutants. Many of the bacterial species have been useful in complementary treatments for the domestic effluents which are rich in lipids and oils. Bacterial lipases have significant industrial, domestic etc. attention because of their stability, substrate specificity, high yielding capacity, and regular supply, as well as the fact that the microorganisms producing them and grow rapidly on inexpensive media. Bacterial biodiversity studies were done using 16S r RNA v3-v4 region of a domestic liquid waste collected from high density and average per capita income ($ 2000) population of eastern India (25°37'19.12'' N, 85° 10' 07.85" E). Next Generation Sequencing (NGS) data set revealed the total 180,933 filtered reads. Analysis of v3-v4 region of filtered reads indicates that species are placed in 9 phyla, 15 classes, 20 orders, 20 families, 20 genus's and 20 species. Among them most dominant phylum was *firmicutes* having 51% diversity and *Chloroflexi* (0.01%) had minimum domination. At the class level *Bacillales* (43%) was dominant, whereas at order and family level bacilli and streptococcus were dominant respectively. *Lactococcus* was most dominant species and surprisingly placed in unclassified group. It has been concluded that the niche of *Janthinobacterium lividum* and *Acinetobacter jonshoni* was isolated and identified in this tropical region. However, *Janthinobacterium lividum* shows the capnophilic behaviour, antitumor properties and its growth favoured by the enhancement of CO_2 concentration. While *Acinetobacter jonshoni* causes human skin infection by colony formation and catheter related blood stream infection. So, we can recommend these bacterial strains to medical as well as in an industrial application for human health concern.

KEY WORDS: BACTERIAL BIODIVERSITY, 16S R RNA, JANTHINOBACTERIUM LIVIDUM, BACILLALES, V3-V4, NGS..

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INTRODUCTION

Bacterial biodiversity is an essential contributor which converts complex organic or toxic components in domestic liquid waste treatment system. As well as these are also essential for the optimal operation and conservation of Biological treatment systems (Moura, 2009). The biological community of activated sludge has a large biological diversity and contains a variety of viruses, bacteria, protozoa, fungi, algae, and metazoan.

In this complex ecosystem, bacteria typically account for 95% of the total number of microbes and play a crucial part in wastewater treatment system.

Nowadays studies of microbiome often give credence to the analysis of 16S ribosomal RNA sequences for the taxonomic identification of bacterial and archaeal strains. Approximately 1600 base pairs long are present on 16S rRNA gene which includes nine hypervariable regions of various conservation (V1-V9) (Handelsman, 2004; Ortiz- Alvarez et al., 2016). More conservative regions are useful for determining the higher-ranking taxa, whereas more quickly evolving ones can help identify genus or species. Metabarcoding using 16S rRNA marker is widespread in the studies of various microbial communities (Bolger et al., 2014; Babilonia et al., 2018).

The introduction of the next-generation sequencing techniques has led to novel applications of metabarcoding methods. In particular, increased read counts have allowed for quantitative estimates of the microbial community composition. Another advantage of NGS-based metabarcoding is that quantitative analysis has become available for communities of uncultured microbes. Yet, using NGS technologies has its limitations, caused chiefly by shorter read length. Its most important impact is the decreased precision of species identification. Domestic liquid wastes contain various type of bacteria and other micro-organisms originating from human wastes and other sources. Many of these bacteria are beneficial and are responsible for the biodegradation of organic components of the wastes whereas some others are may be pathogenic.

Normally bacterial species present in wastewater may cause pathogenicity which infect to the society through food and water. The safe management and disposal of any waste containing human excreta is the most critical aspect of sanitation and hygiene. The sample of domestic waste was collected from bank of river Ganga GPS (Global Positioning System) 25°37'19.12'' N, 85° 10' 07.85" E, the sample collection site is a heart of city, having population density of 1803 per square kilometre and per capita income is 671$ (Cardoso et al. 2017; Babilonia et al., 2018).

Domestic waste treatment system is available only to few percentages of human population. Domestic liquid wastes contain various type of bacteria and other micro-organisms originating from human wastes and other sources. Analysis of bacterial biodiversity in domestic liquid waste water, the variation in bacterial community in domestic liquid waste is temporally, while it mainly effects on human welfare as well as public health (Veldhuis et al., 2010; Taylor et al., 2011). The safe management and disposal of any waste containing human excreta is the most critical, aspect of sanitation and hygiene is essential to prevent the spread of infectious disease. Metagenomic studies gaining importance in providing a better understanding of microbial ecology and its applications in human welfare.

MATRIAL AND METHODS

We collected a domestic liquid waste (pH 8.4) aseptically in sterile glass bottle from house hold discharge near bank of river Ganga specifically identify the bacterial biodiversity. The GPS (Global Positioning System) location of both sample collection site was 25°37'19.12'' N, 85° 10' 07.85" E. Domestic waste water samples were collected and filtered through What-man filter paper No.1 with 0.7 µm retained the average particle size and it was stored at frozen temperature until the analysis.Metagenomic DNA was extracted from the Domestic liquid waste samples by commercially available Nucleospin Soil Kit (Eurofins Genomic lab, Bengaluru, India). The qualities of the isolated metagenomic DNA sample were quantified by using NanoDrop. Sanger sequencing data deposited in GeneBank under the accession numbers PRJNA557310 for bacteria.

Preparation of 2×300 MiSeq library (Illumina MiSeq Next Generation Sequencer) was done using the amplicon libraries were prepared using Nextera XT Index Kit (Illumina inc.) as per the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev.B). Primers for the amplification of the bacterial 16S V3-V4 region were designed and synthesized at Eurofins Genomics Lab. There was approximately 300 base- pair reads generated from each forward and reverse direction which results that entire targeted region having nearly double coverage. Sequence reads were assembled which generates Operational Taxonomic Unit (OTU) that was based on 97% sequence similarity, and two different set of DNAs (chimera) were removed by v 0.38 (Edgar, 2010; Edgar et al., 2011).

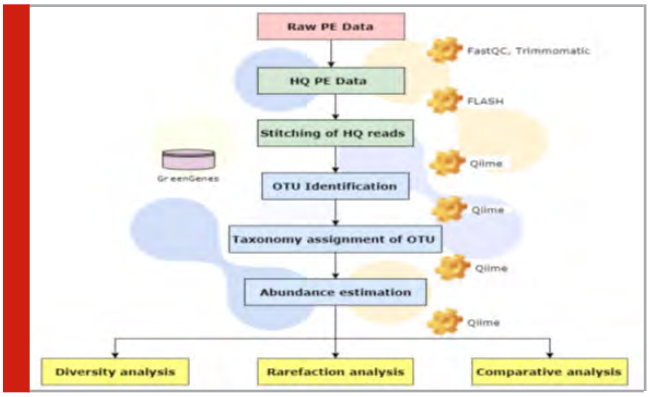
Amplification of the 16S gene was carried out. 3µl of PCR product was resolved on 1.2% Agarose gel at 120V for almost 60 minute or till the samples reached 3/4th of the gel. Primers used in present study was 16S rRNA F-GCCTACGGGNGGCWGCAG and 16S rRNA R- ACTACHVGGGTATCTAATCC. The quality control (QC) passed amplicon with the Illumina adaptor was amplified using i5 and i7 primers that added multiplex index sequence as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon libraries were purified by AMPureXP beads technique and quantified by using Qubit fluorometer. Identified DNA sequences for every OTU were align by using QIIME (Caporaso et al., 2010). Taxonomic distribution of each respective sequence was assigned on the basis of Ribosomal Database Project (RDP) taxonomy (http://rdp.cme.msu.edu).

Quantity and quality check (QC) of library on Agilent 4200 Tape Station was done using only those samples which had mean peak value of amplification of genes obtained through Tape screening method were further used for cluster generation sequencing. The Agilent 4200 Tape Station system was an established automated electrophoresis tool for DNA and RNA sample quality control. Fully automated sample processing enables the unattended analysis of size, concentration and integrity.

The Tape Station system provides a complete solution for true end-to-end sample quality control within any next-generation sequencing (NGS). This covers the full DNA and RNA range and offers high levels of flexibility in a simplified workflow with ready-to-use Screen Tapes. The amplified libraries were analysed on 4200 Tape Station system (Agilent Technologies) using D1000 Screen tape as per manufacturer instructions. D1000 screen tape assay is referred to analysing DNA molecules from 35-1000bp (Ortiz-Alvarez and Casamayor 2016).

Cluster Generation and Sequencing was done after obtaining the mean peak size from Tape Station profile, the libraries were loaded onto MiSeq at appropriate concentration (10-20pM) for cluster generation and sequencing. The MiSeq is an integrated instrument which amplify the clone, sequencing of genomic DNA as well as data analysis with alignment, variant calling, base calling in a single run. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions on MiSeq. The kit reagents were used in binding of samples to complementary adapter oligo on paired-end flow cell.

They were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. The data generated during our study have been deposited in the NCBI (National Centre for Biotechnology Information) Sequence Read Archive (SRA) that are available under the Bio-Sample accession numbers SAMN12393872 and SAMN12393873. The 16S rRNA gene amplicon data generated during our study have been deposited in the NCBI (National Centre for Biotechnology Information) Sequence Read Archive that are available under the Bio-Sample accession numbers SAMN12393872 (Ortiz-Alvarez and Casamayor 2016).



Bioinformatic analysis was done through QIIME (canonically pronounced 'chime'). It is software that performs microbial community analysis. It is an acronym for Quantitative Insights into Microbial Ecology (QIIME), and has been used to analyse and interpret nucleic acid sequence data from fungal, viral, bacterial, and archaeal communities. The QIIME is comprehensive software comprising of tools and algorithms such as FastTree for heuristic based maximum likelihood phylogeny inference (Price Morgan N., et. al., 2010), the Ribosomal Database Project (RDP) classifier for the assignment of taxonomic data using a naïve Bayesian classifier (Wang et al., 2007) and others. This allows QIIME, which continues to undergo development, to easily and relatively adapt standalone tools, and thus improve in step with advances in the field of microbial community ecology (Fig.2) (Ortiz-Alvarez and Casamayor 2016).

RESULTS AND DISCUSSION

The total genomic DNA was isolated from the water-soil mixture using a Nucleospin Soil Kit (Eurofins Genomic lab, Bengaluru, India). The qualities of the isolated metagenomic DNA sample were quantified by using Nanodrop in Table-1.

Table 1. Quantification of Metagenomic DNA in domestic liquid waste

Sample	Nano Drop Reading (ng/µl)	Nano Drop (O.D.A260/280)	Nano Drop (O.D. A260/230)
Domestic liquid waste	127.9	1.82	1.65

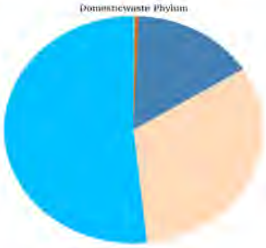
The amplicon library was prepared using a Next era XT index kit (Illumina, Inc.). The primers for the amplification of the V3-V4 region of 16S rRNA genes (forward, GCCTACGGGNGGCWGCAG, and reverse, ACTACHVGGGTATCTAATCC) were designed at Eurofins. The V3-V4 region is highly variable and known to reveal microbially diverse populations. The amplicon library was purified using 1 AM Pure XP beads, quantified using a Qubit fluorometer, and analysed with a 4200 Tape Station system (Agilent Technologies) using D1000 screen tape following the manufacturer's instructions. The library was loaded onto a MiSeq instrument (2 x 300 bp) at 10 to 20 pM for cluster generation and sequencing. The sequenced raw reads were processed to obtain high-quality (HQ) reads using Trimmomatic v0.35 (5) to remove adaptor sequences, ambiguous reads, and low-quality sequences (i.e., those with a more than 5% quality threshold [QV] of <20 Phred quality score). A total of 180,933 high-quality reads were obtained (Yankson and Steck 2009; Ortiz-Alvarez and Casamayor 2016).

The HQ reads were subjected to operational taxonomic unit (OUT) identification at 97% sequence similarity and taxonomic assignment of OTUs using the Green genes database (16S/Archaea database) and the Quantitative Insights into Microbial Ecology (QIIME) module. Krona-based (6) diagram visualization showed that 99.22% of the microbially diverse population represents. When sample of domestic liquid waste were put to Next Generation Sequencing (NGS), large number of bacteria were identified. All of them were grouped into 9 phyla. Out of 9 phyla, *Firmicutes, Proteobacteria* and

Bacteroidetes were most abundant phylum in domestic liquid waste (Ortiz-Alvarez and Casamayor 2016).

Phylum level: At the phylum level bacterial were constitutes *Firmicutes phylum* (51.67%), *Proteobacteria phylum* (32.3%) and *Bacteroidetes phylum* (15.4%) in domestic waste sample (Fig 1). *Betaproteobacteria* was constituents of *Proteobacteria* responsible for degradation of organic matter and nutrient cycling.

Figure 1: Pie chart showing the absolute abundance of the sample at phylum level within each bacterial community. It is shown that the most abundant phylum is *firmicutes*

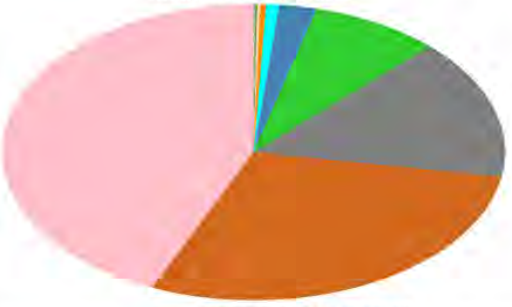


Domesticwaste Phylum legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes	51.67%
	k__Bacteria;p__Proteobacteria	32.3%
	k__Bacteria;p__Bacteroidetes	15.4%
	k__Bacteria;p__Actinobacteria	0.4%
	k__Bacteria;p__Cyanobacteria	0.19%
	k__Bacteria;p__Fusobacteria	0.02%
	k__Bacteria;p__Spirochaetes	0.01%
	k__Archaea;p__Euryarchaeota	0.01%
	k__Bacteria;p__Chloroflexi	0.01%

Identification of bacteria at class level in domestic liquid waste sample through NGS: Now sample were analysed for class level. In which identified bacteria were belongs to 15 classes irrespective of their percentages at phylum level. Out of 15 classes, four classes were dominated and their percentage of occurrence of bacteria as follow *Bacilli* (43.37%), *Gamma proteobacteria* (28.97%), *Bacteroidia* (15.33%) and Clostridia (8.29%) (Fig 2).

Figure 2: Pie chart shows their abundance in percentage of each class within each bacterial community.



Domesticwaste Class legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes;c__Bacilli	43.37%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	28.97%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia	15.33%
	k__Bacteria;p__Firmicutes;c__Clostridia	8.29%
	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria	2.41%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	0.83%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria	0.39%
	k__Bacteria;p__Cyanobacteria;c__Chloroplast	0.19%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	0.09%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia	0.07%
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia	0.02%
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi	0.01%
	k__Bacteria;p__Spirochaetes;c__Spirochaetes	0.01%
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia	0.01%
	Others	0.01%

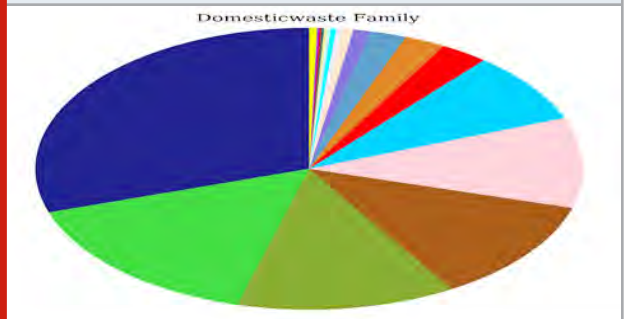
Identification of bacteria at order level in domestic liquid waste sample through NGS: It has been found that *Lactobacillales* was most abundant order of bacteria within each microbial community. The percentage of abundance of bacteria at order level such as *Lactobacillales* (43.22%), *Pseudomonadales* (16.1%), *Bacteriodales* (15.33%), *Enterobacterials* (11.8%) and *Clostridiales* (8.29%) were found in domestic liquid waste (Fig.3).

Figure 3: Pie chart showing the absolute abundance of each order within each bacterial community

Domesticwaste Order legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales	43.22%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales	16.1%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales	15.33%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales	11.8%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales	8.29%
	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales	2.41%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales	1.06%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales	0.69%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales	0.39%
	k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta	0.19%
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales	0.15%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales	0.14%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales	0.07%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales	0.04%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales	0.02%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales	0.02%
	k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales	0.02%
	k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales	0.01%
	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales	0.01%
	Others	0.05%

Figure 4: Pie chart showing the absolute abundance of each family within each bacterial community.



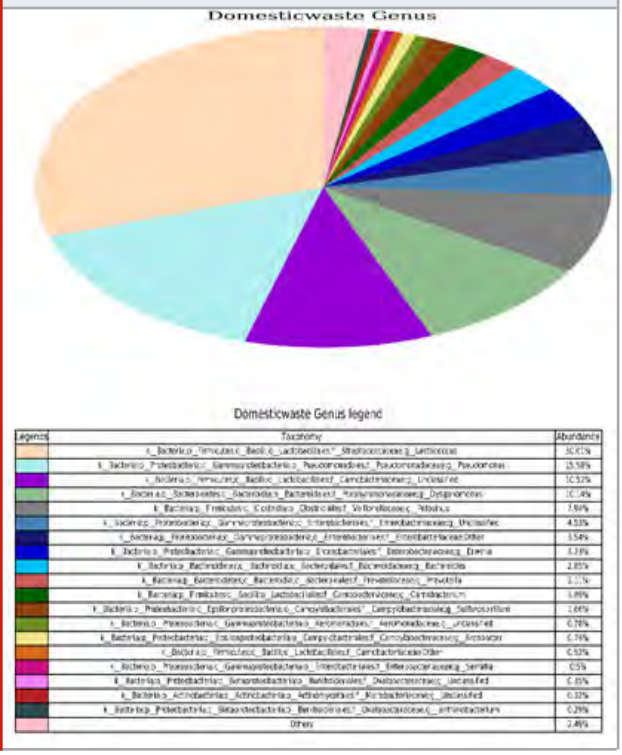
Domesticwaste Family legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	30.01%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae	15.76%
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae	12.94%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	11.8%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae	10.36%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	8.11%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	2.85%
	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae	2.41%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	2.11%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae	1.06%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae	0.65%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	0.34%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae	0.32%
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae	0.21%
	k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__Unclassified	0.19%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae	0.14%
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae	0.13%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae	0.09%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	0.07%
	Others	0.44%

Identification of bacteria at family level in domestic liquid waste sample through NGS: It has been found that *Streptococcaceae* was most abundant family within each microbial community in taxonomic distribution of domestic waste. The percentage of abundance of bacteria at family level were *Streptococcaceae* (30.01%), *Pseudomonadaceae* (15.76%), *Carnobacteriaceae* (12.94%), *Enterobacteriaceae* (11.8%), *Porphyromonadaceae* (10.36%) and *Veillonellaceae* (8.11%) (Fig.4).

Identification of bacteria at genus level in domestic liquid waste sample through NGS: It can be inferred that the most dominated genus was *Lactococcus* in domestic waste. The absolute abundance of each genus within each microbial community were *Lactococcus* (30.01%), *Pseudomonas* (15.58%), *Unclassified* (10.52%), *Dysgonomonas* (10.14%), *Pelosinus* (7.94%) (Fig.5) (Ortiz-Alvarez and Casamayor 2016).

Figure 5: Pie chart showing the absolute abundance of each genus within each bacterial community. From the figure, it can be inferred that the most abundant genus is *Lactococcus*



Identification of bacteria at species level in domestic liquid waste sample through NGS: It has been found that the most abundant bacteria at species level taxonomic distribution was unclassified bacteria but at genus level it belongs from *Lactococcus*. The percentage of abundance within each bacterial community were Unclassified from *Lactococcus* genus (30.01%), Unclassified from *Pseudomonas* genus (15.29%), Unclassified from Carnobacteriaceae family (10.52%), Unclassified from *Dysgonomonas* genus (10.14%) (Fig.6) (Yankson and Steck 2009).

It can be inferred that the most dominated genus was *Lactococcus* in domestic waste. The absolute abundance of each genus within each microbial community were *Lactococcus* (30.01%), *Pseudomonas* (15.58%), Unclassified (10.52%), *Dysgonomonas* (10.14%), *Pelosinus* (7.94%). The data representing *Firmicutes* (0.05088%), *Proteobacteria* (0.2445%), and Bacteroidetes (0.148%), was classified to the species level (Table 2) (Yankson and Steck 2009).

Figure 7: Pie chart showing the absolute abundance of each species within each bacterial community.

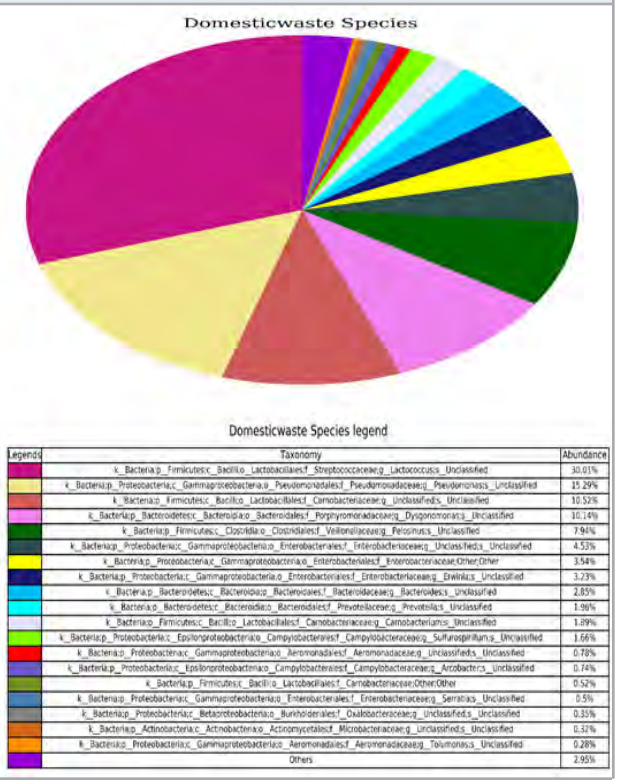
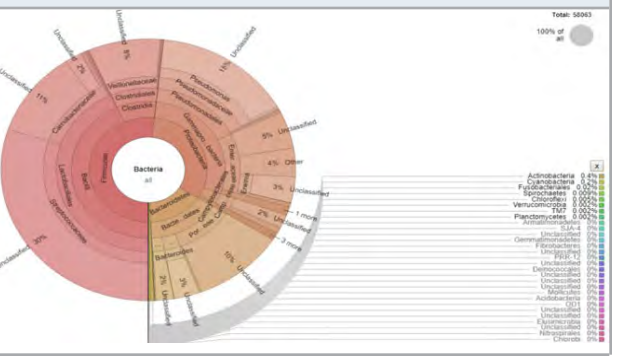


Figure 8: Krona chart of the bacteria represented by 16S rRNA gene amplicon-based bacterial diversity in domestic liquid waste. Each circle represents the phylum, class, order, family, genus, and species from the inside to the outside of the circle, respectively, indicated by percent diversity based on the absolute number of representative bacteria



Our study site revealed that the partially treated domestic liquid waste, which also creates a carbon source of microbes and results is huge bacterial diversity. In our knowledge, very little or no explicit work on flood plain bacterial biodiversity by Next Generation Sequencing (NGS) is available. So, we attempted this study to reveal bacterial biodiversity in such a complex ecosystem/ niches by NGS. The software of Illumina-based, as per the 16S Metagenomic Sequencing Library preparation protocol, near full-length and differential diversity studies. Metagenomic DNA was isolated from the collected sample of domestic liquid waste (DLW).

Table 2. Summary of bacteria identified to the species level from 16S RNA gene- based metagenomic study of a domestic liquid waste collected from near to PMCH, Patna Bihar, India

Phylum	Class	Order	Family	Genus	Species	Absol. Count	%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*Johnsonii*	142	0.2445
	Betaproteobacteria	*Burkholderiales*	*Oxalobacteraceae*	*Janthinobacterium*	*Lividum*	*138*	0.2377
	Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*Viridiflava*	*76*	0.1309
	Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*Fragi*	*70*	0.1206
	Gammaproteobacteria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*rhizosphaerae*	*5*	0.0086
	Gammaproteobacteria	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	*Sanguinis*	*3*	0.0052
	Alphaproteobacteria	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	*Adhaesivum*	*3*	0.0052
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*Copri*	*83*	0.1429
	Bacteroidia	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*Stercorea*	*2*	0.0034
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Faecalibacterium*	*Prausnitzii*	3	0.0052
	Clostridia	*Clostridiales*	*Veillonellaceae*	*Veillonella*	*Dispar*	1	0.0017
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Collinsella*	*Aerofaciens*	3	0.0052
	Actinobacteria	*Actinomycetales*	*Micrococcaceae*	*Rothia*	*mucilaginosa*	1	0.0017
	Actinobacteria	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*	*Variabile*	3	0.0052
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Akkermansia*	*Muciniphila*	1	0.0017
Fusobacteria	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Cetobacterium*	*Somerae*	2	0.0034

Extraction of DNA is a vital step because it can affect on both the quality and quantity of DNA extracted. Furthermore, an increase the concentration of DNA yield is important to ensure that the DNA sample is representative of the gene pool (A gene pool is the collection of total genes within an interbreeding population) of the collected sample. The extraction of DNA yield may be depending on the physical properties and chemical composition of the matrix. The concentration of quantified DNA was found in domestic liquid waste as 127.9 (ng/µl) by Nano drop readings which may indicate the more bacterial diversity in their community (Zhou et al., 1996; Lakay et al., 2007; Yankson and Steck 2009).

Inspite of that the amount of extracted DNA purity of examined by the ratios A260/280 and A260/230 and the integrity of the extracts checked by agarose gel electrophoresis, are also important parameters for gene amplification by PCR (von Wintzingerode et al., 1997). When the ratio of A260/230 is low that indicates the protein contamination in DNA extraction from the samples which reflected the co-extraction of contaminants absorbing at 230 nm such as residual phenol from nucleic acid extraction and humic acid and fulvic acids (Whitehouse and Hottel 2007; Techer et al., 2010). So, in our sample we got quantification of isolated metagenomic DNA sample on nanodrop was the ratio of A260/230 -1.65 in domestic liquid waste.

The sequenced library was further analyzed through bioinformatics tools. There were used a software for the data analysis called as QIIME software. QIIME (canonically pronounced 'chime') is software that performs microbial community analysis. It is an acronym for Quantitative Insights into Microbial Ecology (QIIME), and has been used to analyses and it also interpret nucleic acid sequence data from fungal, viral, bacterial, and archaeal communities. Operational Taxonomic Unit (OTUs) are clusters of sequences, frequently intended as 97% sequence similarity. Bioinformatics studies and taxonomic profiling revealed existence complex of bacterial population at the phylum level comprising of *Frmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, Chloroflexi* etc. in domestic liquid waste.

From the analysis, it was conferred that the phylum Firmicutes (51.67%), was most abundant in domestic liquid Waste. samples exhibited high degree of diversity at class level. The prominent communities identified were *Bacilli, Gamma proteobacteria, Bacteroidia, Clostridia, Alpha proteobacteria, Delta proteobacteria, Thermoleophilia, Planctomycetia* etc. *Bacilli* (43.37%). Study also revealed presence of great diversity at the order level such as *Lactobacillales, Pseudomonadales, Bacteriodales, Enterobacteriales, Clostridiales, Actinomycetales, Bacillales, Rhizobiales* etc. in domestic liquid waste, Out of these identified bacteria at class level, *Lactobacillales* (43.22%). We have found that *Streptococcaceae* (30.01%)

was relatively most abundant bacteria at phylum level of taxonomic distribution in DLW.

Unclassified bacteria at the genus level were found in both samples. Unclassified belongs to genus belongs to *Carnobacteriaceae* (10.52%), *Nocardioidaceae* (17.53%) and *Micrococcaceae* (5.62%) family. After that Alpha diversity summarizes the diversity of organisms in a sample with a single number. This diversity of annotated samples can be estimated from the distribution of the species – level annotations. Shannon alpha diversity of domestic waste was 4.822. Our study revealed previously unknown groups of bacteria highlighting the continued importance of general culture independent 16S rDNA surveys of unexplored environments. These novel bacteria make up a substantial proportion of the diversity sampled and are likely to play key roles in Himalayan soil biogeochemistry.

CONCLUSION

This study provides essential baseline information on bacterial communities in the domestic liquid waste (near PMCH, Bihar). The most abundant identified bacterial biodiversity from phylum to species level in the domestic liquid waste. The relative abundance of bacterial diversity differed significantly according to the DNA extraction method used; so, the results of studies using different methods need to interpretation. This result will be the selection of an appropriate DNA extraction for metagenomics studies which help to st6 different toxic as well as.

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Conflict of Interest: The author has no conflict of interest.

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Methanolic Extract of *Phyllanthus niruri* Ameliorates Certain Biochemical and Metabolic Changes in Streptozotocin-Induced Diabetic Mice

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ABSTRACT

Diabetes mellitus metabolic disorder with impaired carbohydrate, fat, and protein metabolism characterized by increased blood glucose levels. Diabetes being the multifactorial disorder is represented with various organ and metabolic irregularities. Of all those, irregularities of Liver, Kidney, Pancreas, and lipid metabolism are common. Rosiglitazone, thiazolidinediones which are a synthetic drug having various side effects was used for comparative analysis with the desired botanical extract which is safe, having lesser or no side effects and less expensive. Based on folkloric usage and reported literature the present study aimed to investigate the whole plant extract of *Phyllanthus niruri* Linn. (Euphorbiaceae) for biochemical, enzymatic, and enhanced glucose utilization properties on *Swiss albino* mice. The mice were divided into five groups namely, STZ Induced diabetic control mice (35mg/ kg. body weight) non-diabetic control mice, Diabetic treated (DT150) (150 mg/ kg of body wt. extract), Diabetic treated (DT250) (250 mg/ kg of body wt. extract), Diabetic treated (DTRGZ) (2mg/ kg of body wt. rosiglitazone). The Tukey–Karmer Post Hoc test was applied to identify significance among groups. Graphs are plotted using MATLAB version 7.8.0. The extract of *Phyllanthus niruri* showed better outcomes and the result obtained was statistically significant. The result showed better restoration of those biochemical parameters like Glucose, ALT, AST, urea, creatinine, and lipid profiles. It was found that the extract of 250mg/kg. body weight was more effective than other groups and hence the research recommends the exogenous use of the *P. niruri* could be the best candidate to regulate the diabetic complications however, translational research with a large sample size is required.

KEY WORDS: DIABETES MELLITUS, STREPTOZOTOCIN, PHYLLANTHUS NIRURI, SWISS ALBINO, ROSIGLITAZONE.

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INTRODUCTION

The human population has always been plagued by diseases that have adversely affected their health and well-being. Of these, one particular disease that causes greater morbidity and mortality, in both young and old people is Diabetes mellitus accompanied with macro vascular and micro vascular complications. It is a metabolic disorder with impaired carbohydrate, fat, and protein metabolism characterized by increased blood glucose levels (Skyler et al., 2017). Statistical analysis of diabetes represents that there are about 463 million

adults having age between 20-79 years who are suffering from this disease which is projected to increase by 700 million by 2050. Its propensity is increasing in low and middle-income countries which are 3 in 4 (79%) are diabetes sufferers. It is estimated that 1 in 5 people (136 million) of age above 65 years are having diabetes and 232 million are undiagnosed (IDF, 2019). Diabetes caused 760 billion USD expenditure in 2019 which is 10% of the total health expenditure. More than 20 million live births (1 in 6 births) are affected by hyperglycemia and out of this 84% developed gestational diabetes mellitus. 764 million candidates are at increased risk of developing diabetes globally (IDF, 2019).

Diabetes being the multifactorial disorder is represented with various organ and metabolic irregularities. Of all those, irregularities of the Liver, Kidney, Pancreas, and lipid metabolism are common. It is associated with hepato-renal and lipid abnormalities which are reflected by altered organs and its metabolic activities (Sirovina et al., 2016). Metabolic diseases like Non-alcoholic fatty liver disease (NAFLD) are the most common which accounts for 70-80% diabetes sufferer. The co-existence of Diabetes and NAFLD sometimes causes serious consequences leading to a severe form of NAFLD and chronic vascular complications of Diabetes mellitus (Targher et al., 2018; Elhence et al., 2020).

Recent research conducted on human and animal models strongly supports the concept that the potent reason for diabetic nephropathy is associated with altered metabolism of glucose and persistent hyperglycemia. Oxidative stress is known to play a significant role in the induction of these abnormalities (Miranda-Diaz et al., 2016). High levels of oxidative stress with the excessive generation of free radicals and depleted levels of free radical–scavenging enzymes have been demonstrated in several studies, both in experimental animal models and in humans with diabetes (Rahman et al., 2017; Yaribeygi et al., 2020).

It had been shown that the presence of diabetes encourages enhanced cardiovascular diseases (CVD) risk as compared to non-diabetes with marked complications like heart failure, peripheral arterial disease, and coronary heart disease (Glovaci et al., 2019). Patients with type 2 diabetes (T2D) show the widespread prevalence of lipid abnormalities, contributing to cardiovascular diseases. Diabetic dyslipidemia includes quantitative, qualitative, and kinetic lipoprotein abnormalities, which, altogether, cause an imbalanced atherogenic lipid profile (Gupta et al., 2019).

The market is flooded with different kinds of mono target drugs with good potential. In spite of excellent vigor, these synthetic anti-diabetic drugs had presented undesirable therapeutic reputation marked with fluid retention, hypoglycemia, liver hepatic issues, lactic acidosis, weight gain, and cardiac hypertrophy. Rosiglitazone, a thiazolidinedione in the present study was used for comparative analysis with the desired botanical extract. It behaves widely as an insulin sensitizer by altering the transcriptional activity of PPARγ. Meanwhile, Food and Drug Administration (FDA) had severely restricted its use because of weight gain, fluid retention, bone fractures, and the associated increase in congestive cardiovascular complications (Kahn et al., 2010; Lebovitz et al., 2019).

Thus, a collaborative translational effort for the search of more effective medicine for T2DM has become the need of the time regarding the safety and efficacy due to the unavoidable side effects of synthetic drugs. For example, metformin, less toxic biguanides, and the potent oral hypoglycemic agent was developed from the *Galega officinalis* plant and used to regulate hyperglycemia. Based on folkloric usage and reported literature the present study aims to investigate the whole plant extract of *Phyllanthus niruri* Linn. (Euphorbiaceae) for Biochemical, enzymatic, and enhanced glucose utilization properties. Earlier it was showed that the extract of this plant has strong anti-oxidative and anti-diabetic properties due to the presence of active phytochemical components like polyphenols, tannin, and flavonoid. *In-vitro* and *in-vivo* studies have provided scientific evidence, whereby administration of *P. niruri* leaf aqueous extract to diabetic rats reduced oxidative burden in the kidney by preventing the depletion of endogenous antioxidant enzymes (Swapna et al., 2014). In a recent study, the cardioprotective action of aqueous extracts of *P. amarus* was studied against high-sugar (fructose) diet-mediated cardiac damage in Wistar rats (Bailey et al., 2017).

Following 60-days of sugar diet, and Co-administration of *P. amarus* aqueous extracts for 60 days impeded cardiac and aortic lipids profile and decreased phospholipid formation (Putakala et al., 2017). The aqueous extracts of *P. niruri* were extensively studied and it was found that due to the presence of phenolic components in the extract displayed the anti-diabetic properties (Singh et al., 2017). In another study with the extract of *P. niruri* demonstrated that it lowers the Diabetic complications like obesity, oxidative stress by decreasing glucose concentration which decreases glycosylation and hence decreased oxidative stress and lipid levels in the blood of obese diabetic rats (Mediani et al, 2016). Recent research evidences *In vivo* administration of aqueous extract of *P. niruri* in rats (25, 50, 100 and 200 mg/kg) caused normalization of AST, ALT, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total cholesterol (TC), triglycyrides (TG), total bilirubin (TB), glucose, total proteins (TP), urea and creatinine levels which were elevated by carbon tetrachloride (Ezzat et al., 2020). Based on the previous studies a comprehensive study was designed to trace the efficacy of the synthetic drugs and botanicals synergistically.

MATERIAL AND METHODS

This study includes whole plant extract of *Phyllanthus niruri* Linn. (Euphorbiaceae) which was collected from Science College Campus of Patna University, Patna, Bihar, India. It was further identified and botanically authenticated according to the relevant monographs

of Indian Pharmacopoeia (2012) and the same has been deposited in the Bio-chemistry Department, Patna University, Patna, Bihar, India. For animal studies, the research study was carried out on the albino mice weighing around 16–20 gram having 6.4 ± 0.5 cm lengths. The mice were housed in polypropylene cages and fed on normal lab made chow and maintained under standard environmental conditions (21±2°C, 55±5% humidity, 12 hr Light: Dark cycle).

To study the induction of diabetes, the Streptozotocin (STZ) is purchase from Merck was dissolved in 1mMolar citrate buffer at the pH 4.5 and always prepared fresh for immediate use (within 5 minutes). Diabetes was induced by multiple intra-peritoneal injection of freshly prepared STZ solution in 0.05 M sodium citrate (pH 4.5) at the dose of 35 mg/kg body weight followed by fasting (Andrade et al., 2016).

To study the animal groupings and experimental design, the mice were divided into five groups having six mice in each group:

Group – I STZ Induced diabetic control mice receiving citrate buffer only
Group II non-diabetic control mice receiving only citrate buffer solution
Group III Diabetic treated (DT150) receiving of 150 mg/ kg of body wt. extract
Group IV Diabetic treated (DT250) Receiving 250 mg/ kg of body wt. extract
Group V Diabetic treated (DTRGZ) Receiving 2mg/ kg of body wt. Rosiglitazone

For the extract preparation, freshly harvested plant samples were washed under running tap water, blotted with filter paper and was dried in the shade at room temperature. The dried plant sample (2.6 kg) was then soaked with absolute methanol under reflux condition for the methanolic extract preparation. The sample was then homogenized with extraction buffer and the supernatant collected after three rounds of extraction. The solvent was evaporated under reduced pressure in a rotary evaporator at 40 ^{0}C. To this thick paste colloidal silicon dioxide was added and dried in vacuum tube dryer. The obtained plant extract was stored in freezer at -20^0 C until further test.

For the Biochemical estimation, the desired Biochemical parameters were accessed to monitor the metabolic activity of the mice in the respective groups.Fasting Plasma Glucose by GOD POD method (Trinder, 1969), serum Cholesterol CHOD POD , triglyceride uding GPO method, HDL by Phosphotungestic method (Burstein et al.,1970). Serum LDL and VLDL were calculated using Frieldwald formula, serum creatinine by alkaline picrate method (Jaffe, 1986), Serum urea by Nitroprussic method, Alanine aminotransferase (ALT) Reitman and Frankel method and Aspartate aminitransferases (AST) Modified IFCC method (Schumann et al., 2002).

For the statistical analysis, data were expressed as the mean ± S.E.M. For statistical analysis of the data, group means were compared by one-way ANOVA (analysis of variance) with *Post Hoc* analysis. The Tukey–Karmer Post Hoc test was applied to identify significance among groups. Graphs are plotted using MATLAB version 7.8.0 R2009a, Natick, Massachusetts: The Mathworks Inc. 2009.

RESULTS AND DISCUSSION

Table 1. Body weight changes in mice

Groups	Day 0	Day 7	Day 15
Normal control (NC)	18.95 ± 2.76	20.80 ± 2.39	23.89 ± 2.20
Diabetic control (DC)	10.70±1.05	09.45±0.95	8.47±1.32
P. niruri extract (150 mg/kg) (DT150)	10.71±2.03*	12.11±1.67*	13.72±1.53*
P. niruri extract (250 mg/kg) (DT250)	10.68±1.63*	12.74±2.37*	14.84±2.67*
Rosiglitazone (2 mg/kg) (DTRGZ)	10.70 ± 3.74*	14.82 ± 3.91*	16.04 ± 1.84*

Values expressed as Mean ± S.E.M, n = 6 in each group; *Significant as compared to control.

Effect of P. niruri extract on body weight: The diabetic control (DC) mice presented significantly lower body weight ($p<0.001$) when compared with the normal control (NC) mice which indicates towards the stress condition of the mice (Xu et al., 2017). A significant body weight gains were observed in the treated groups of diabetic mice (DT150 and DT250) as compared to the DC ones. The DT150 and DT250 group showed an increase of 28% and 39% in body weight respectively after 15 days of treatment. Contrary to this, DTRGZ group mice showed

an increase of 50% in body weight after 15 days of treatment (Table 1). Extract of the *P. niruri* reduced the diabetic complication and reordered the metabolic activity in mice resulted into restoration of the body weight (Wat et al., 2018).

Effect of P. niruri extract on blood glucose level: The changes in the blood glucose levels before and after receiving the treatment in normal and diabetic mice are listed in Table. As expected, the DC mice showed significantly ($p<0.001$) higher level of glucose (+278%), when compared with their normal control counterparts. Diabetic mice of both of the groups (DT150 and DT250) showed a reduction in glucose levels when compared to the DC ones; nevertheless, the reduction was particularly evident in the DT250 mice (–50%; $p<0.001$).

Table 2. Effects of different doses of *P. niruri* extract and rosiglitazone on blood glucose levels in mice.

Groups	Blood glucose levels (mmol/l) in week				
	Pretreatment	Post-treatment			
	0	1	2	3	4
Normal control (NC)	3.95 ±0.13**	4.07 ± 0.14**	4.06 ± 0.25**	4.05 ± 0.16**	3.99 ± 0.19**
Diabetic control (DC)	14.96 ± 1.55*	14.91 ± 1.48*	14.78 ± 1.59*	14.96 ± 1.49*	14.94 ± 1.48*
P. niruri extract (150 mg/kg) (DT150)	14.97 ± 1.40	13.04 ±1.18*	10.64 ±2.09**	9.69 ± 1.28**	9.27 ± 1.79**
P. niruri extract (250 mg/kg) (DT250)	14.64 ± 1.59	11.86 ± 1.38**	9.65 ± 1.28**	8.27 ± 1.74**	8.15 ±1.28**
Rosiglitazone (2 mg/kg) (DTRGZ)	15.03 ± 1.49	9.84 ± 1.48**	5.57 ± 1.28**	4.97 ± 1.35**	4.94 ± 0.97**

* $p<0.05$ as compared with normal control. ** $p < 0.01$ as compared with diabetic control.

Table 3. Effects of different doses of *P. niruri* extract and rosiglitazone on serum lipid levels in mice.

Groups	TC (mmol/lit)	TG (mmol/lit)	HDL (mmol/lit)	HDL/TC (%)	LDL (mmol/lit)
Normal control (NC)	4.15 ±0.86**	1.14 ±0.09**	2.86 ±0.29**	68.91 ±4.66**	0.27 ±0.04**
Diabetic control (DC)	9.84 ±1.56*	1.96 ±0.29*	1.31 ±0.58*	13.31 ±1.97*	0.96 ±0.16*
P. niruri extract (DT150) (150 mg/kg)	7.23 ±0.44**	0.87 ±0.08**	2.54 ±0.36	35.13 ±3.37**	0.47 ±0.06**
P. niruri extract (250 mg/kg) (DT250)	6.42 ±0.64**	0.98 ±0.17**	2.78 ±0.46**	43.39 ±4.26**	0.44 ±0.07**
Rosiglitazone (2 mg/kg) (DTRGZ)	6.58 ±1.35**	1.77 ±0.17**	2.90 ±0.55**	44.07 ±5.56**	0.41 ±0.09**

* $p<0.05$ as compared with normal control. ** $p < 0.01$ as compared with diabetic control.TC-total cholesterol, TG-total glycerol, HDL- high density lipoprotein, LDL-Low density lipoprotein.

When compared, the glucose levels of the DT250 versus the DC group mice during the 4-week treatment program, a significantly lower value in the first was also found (–68%; $p<0.001$). Nevertheless, this drop in the glucose levels was more evident in the DT150 rats (–57%) than in the DT250 mice. In contrast to this, DTRGZ group mice showed almost 100% drop in glucose level after 4-weeks of the treatment program (Table 2). These findings of the botanical extract of *P. niruri* may be indebted to their blood glucose-lowering properties to inhibition of glucose absorption and enhancement of glucose storage and utilization. The findings are in line with the previous study (Okoli et al., 2011; Thakur et al., 2016).

Effect of P. niruri extract on lipid profile: When compared with normal control, the diabetic mice had higher total cholesterol (TC) (+137%; $p<0.001$) and TGs (+72%; $p<0.001$) values (Table 3). These changes in biochemical parameters are as expected, as when the uncontrolled diabetic status progresses, substantial changes in total

cholesterol and triglycerides values are predictable. Diabetic mice treated with lower dose of *P. niruri* extract (DT150) showed significantly lower values of serum TC (-26.5%; p<0.001) and TGs (-55.6%; p<0.001), when compared with the DC counterparts. The DT250 treatment showed superior lowering effects compared with the DC counterparts as well as DT150 group mice by (-34.7%; p<0.001) on serum TC levels and (-50%; p<0.001) on TGs levels (Table). Contrarily, treatment with rosiglitazone (DTRGZ) showed (-33.2%; p<0.001) on TC levels and (-09.6%; p<0.001) on TGs levels compared with diabetic control mice (Table 3).

Relative to normal control, the diabetic mice had higher value of low-density lipoprotein (LDL) (+250%; p<0.001) while diminished value of high-density lipoprotein (HDL) (-54%; p<0.001) (Table). This is because when the unrestrained diabetic condition advances, considerable changes in these biochemical parameters are as expected and predictable. Diabetic mice treated with lower dose of *P. niruri* extract (DT150) showed significantly lower values of serum LDL (-62.1%; p<0.001) and higher value of HDL (-48.4%; p<0.001), when compared with the DC counterparts (Table).

All over again, the DT250 treatment showed even better lowering effects on LDL (-34.7%; p<0.001) compared with the DC counterparts as well as DT150 group mice and improved level of HDL (+52.9%; p<0.001) (Table). In contrast, treatment with rosiglitazone (DTRGZ) showed a considerable diminished level of LDL (-175%; p<0.001) while improved level of HDL (+70.5%; p<0.001) compared with diabetic control mice (Table 3). The explanation for the present investigation is that *P. niruri* may prevent hyperlipidemia by decreasing lipid accumulation and the finding is in consistent with (Mediani et al., 2016).

Table 4. Blood urea, creatinine, ALT & AST in all groups before and after treatment with *P. niruri* extract.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	ALT (IU/L)	AST (IU/L)
Normal control (NC)	38.15 ± 0.44**	0.89 ± 0.038**	28.47 ± 0.48**	67.13 ± 1.43**
Diabetic control (DC)	90.45 ± 1.73*	1.48 ± 0.037*	63.75 ± 1.74*	112.42 ± 2.43*
P. niruri extract (150 mg/kg) (DT150)	48.22 ± 1.15**	1.24 ± 0.054**	32.85 ± 2.94**	63.48 ± 0.75**
P. niruri extract (250 mg/kg) (DT250)	37.79 ± 2.36**	1.06 ± 0.026**	27.74 ± 0.55*	63.87 ± 1.45*
Rosiglitazone (2 mg/kg) (DTRGZ)	38.57 ± 0.15	0.97 ± 0.025	30.94 ± 0.57	73.47 ± 1.76

* p<0.05 as compared with normal control. ** p < 0.01 as compared with diabetic control.

Effect of P. niruri extract on kidney function and liver function markers: Diabetic mice have higher levels (approximately twice) of blood urea, creatinine, SGOT, SGPT. All four markers decrease considerably in Rosiglitazone treated diabetic mice (DTRGZ) when compared to diabetic control mice (DC). In DTRGZ mice the parameters, serum urea, serum creatinine, serum SGPT, and serum SGOT were reduced by 134%, 52%, 106%, and 53% respectively (Table 4) Treatment with *P. niruri* extract decreases the values of all the four markers in a dose-dependent manner when compared to diabetic control mice.

The maximum efficacious dose was found to be 250 mg/kg body weight of mice (Table 4). Thus, the result showed that the *P. niruri* extract is also as effective as rosiglitazone in improving kidney function. The extract of *Phyllanthus niruri* helps to preserve kidney function towards normal by ameliorating histopathological changes through reduction of, inflammation, fibrosis, and apoptosis in diabetic mice. The present results corroborate with the previous findings (Giribabu et al., 2017).

CONCLUSION

The plant under investigation, *P. niruri* whole plant extract is anti-diabetic due to the presence of different types of active components, which may have different mechanisms of action which reflects with the restored concentration of glucose, organ function enzymes, and lipid level. Treatment of diabetic albino mice with the two different doses of *P. niruri* extracts showed a dose-dependent differential protective effect on liver function tests and kidney function tests. Extract of *P. niruri* also reduced the secondary complications differentially, including cardiovascular diseases, insulin resistance, and atherosclerosis caused due to hyperlipidemia in diabetic rats. Therefore, relying on botanical phytochemicals as an anti-diabetic therapy may be beneficial and this could be considered as a safe supplementary therapy for long-term and effective management of diabetic patients.

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An Optimal Feature Selection with Whale Algorithm and Adaboost Ensemble Model for Pancreatic Cancer Classification in PET/CT Images

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ABSTRACT

Pancreatic cancer causes the fourth most cancer-related death in humans worldwide. Early detection of this cancer will improve patient's survival rate considerably. In this paper, we propose an image processing and machine learning system for the exact recognition of pancreatic cancer using PET/CT scan images. The proposed system implicates 5 main elements, i.e., preprocessing, segmentation, feature extraction, feature selection (optimization) and classification. Removal of noises in the image is the major step for the exact identification of tumor, if noises persist; it will provide an in-accurate result. Pre-processing is done as an initial step in removing the noises followed by segmentation in identifying the tumor location; here a novel approach of saliency-based k-means clustering algorithm is utilized to isolate the object from background. Since the features extracted from segmented images consist of irrelevant features, it reduces the classification accuracy in disease recognition. So, efficient feature selection method is introduced in this research work to improve the classification performance. To improve feature selection results, initially, image segmentation is carried out by using saliency-based k-means clustering segmentation, and then feature extraction is done by using First Order and Second Order Statistical features by GLCM and GLRM. Feature selection methods such as PSO and whale optimization methods are utilized. The results obtained by these methods indicate the potential advantages of using feature selection techniques to improve the classification accuracy with a smaller number of feature subset. From the result, one can conclude that the performance of whale is superior to PSO method for classification. Machine Learning Techniques are widely used for the cancer classification. The machine learning classifiers such as DT, KNN, SVM and AdaBoost with ensemble KNN - SVM classifier are utilized to classify the tumor as normal or abnormal. Finally, the proposed framework achieves a classification accuracy of 98.3%.

KEY WORDS: ADABOOST, ENSEMBLE, PANCREATIC CANCER, PET/CT, WHALE OPTIMIZATION ALGORITHM (WAO).

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INTRODUCTION

Pancreatic cancer is the 9th typical cancer in women then the 10th typical cancer in men and 4th most cancer related death worldwide. It reasons for '7%'of all cancer deaths. There are currently no strategies for preventing pancreatic cancer, so early recognition of this syndrome is a major aspect and plays an important role in reducing

death rate. Interpreting the PET/CT scan image is the finest approach to assess early on the existence of pancreatic cancer. However, various studies have shown that in addition of having high rates of false positives, physicians can miss the recognition of a substantial part of abnormalities. Feature extraction plays a significant role in classifying the disease in pancreatic cancer detection. Transformation of the segmented image into a set of features are called Feature Extraction. The block diagram for the proposed experimentation is shown below (Shah et al., 2015).

Pre-processing stage in this system is to eliminate the noise and artifact present in the image. Next, K-Means Clustering based on saliency is used to segment the tumor area that is situated on a non-uniform basis. GLCM and GLRM perform feature extraction technique after the segmentation. A Whale Optimization Algorithm (WAO) technique was then proposed for selecting the best features and is compared with particle Swarm optimization (PSO) for selecting the features. Finally, Using the Adaboost with Ensemble KNN-SVM classifier, all the extracted features are classified as normal tumor or abnormal tumor.

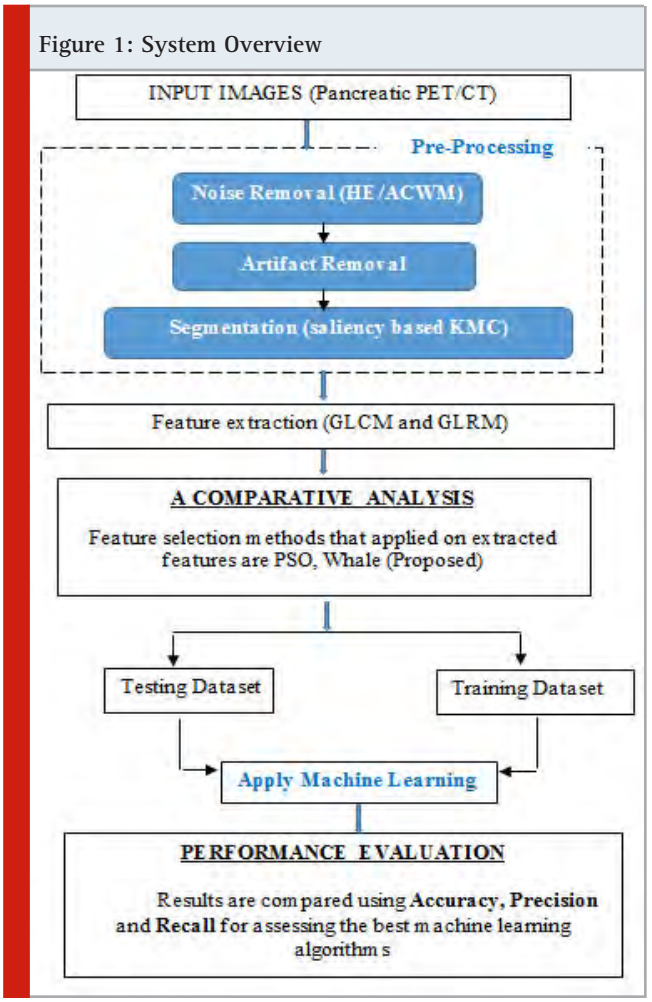
This section highlights the various techniques applied to identify the pancreatic tumor. Pancreatic cancer can be identified at an early stage by preprocessing the CT images using median filter and classified by minimum distance classifier and achieved the accuracy of 65% as stated by (Shah et al., 2015). Sheelakeshvan et al., (2017) suggested that 60% accuracy can be achieved during classification. In the early stages from CT scans, pancreatic cancer can be diagnosed by using different filters such as Median, Gaussian filters with Image segmentation and Artificial Neural Network (ANN) classifiers (Akhtar, Gupta and Ekbal, 2017).

Balakrishna et al., (2018) proposed the computer aided diagnostic model for pancreatic tumor in CT scan images. Median, Gaussian and Wiener noise filtering methods are applied on CT images for preprocessing. Comparing other filters, Wiener provides best results based on certain metrics such as PSNR (Peak Signal Noise Ratio), SNR (Signal Noise Ratio) and MSE (Mean Square Error). SFTA (Segmentation-based Fractal Texture Analysis) approach utilized for extracting the features from objects. For classification system, several machine-learning algorithms are applied but SVM classifier produces results that are more relevant. Li and Jiang (2019) proposed a CAD model, in which simple linear iterative clustering (SLIC) is performed for segmentation, PCA (Principal Component Analysis), is developed for feature selection, and finally (HFB-SVM-RF) approach is aimed to identify the normal and abnormal pancreatic cancer and achieved the accuracy of 96 % (Sarangi, Samal and Sarangi, 2019).

MATERIAL AND METHODS

The research work is implemented on MATLAB software tool and utilized as a user-friendly interface. For the experimental tests, the PET/CT image dataset is used in this framework. The architecture proposed consists of models of pre-processing, removal of artifacts, segmentation, Feature Extraction (FE), Feature Selection (FS) and Classification (Balakrishna and Anandan, 2018). It is described in the below figure. This Model is designed to help the radiologist reliably in identifying the abnormalities in pancreatic cancer.

Figure 1: System Overview



For preprocessing, in a digital image, a pixel's brightness characteristics have an effect on background noise and therefore image pre-processing becomes necessary. Improving the image quality and making it ready for further processing by eliminating the unnecessary noises and artifacts in the PET/CT context is the main goal of image pre-processing system. Figure 2, shows the pre-processing stage, where the Median based filter that is a ' nonlinear' noise filter type is applied to minimize the input image noise effects without blurring the edges (Shah and Surve, 2015).

The process of this filter is to arrange the pixel values in any order (ascending or descending) and then to calculate the center weighted median value and to replace the noisy pixel with that value. In addition, HE (Histogram Equalization) that is an Image Processing technique utilized for enhance the image contrast. The Image enhancement consists of creating the visual illusion,

that the image is more realistic for machine vision applications. Hence, noise are removed by Histogram Equalization/ Adaptive Center Weighted Median filter (HE/ACWM) and removing the letter artifacts by Combination of Standard Deviation and Computational Geometry Technique (Ali et al., 2020).

Figure 2: Image pre-processing steps.

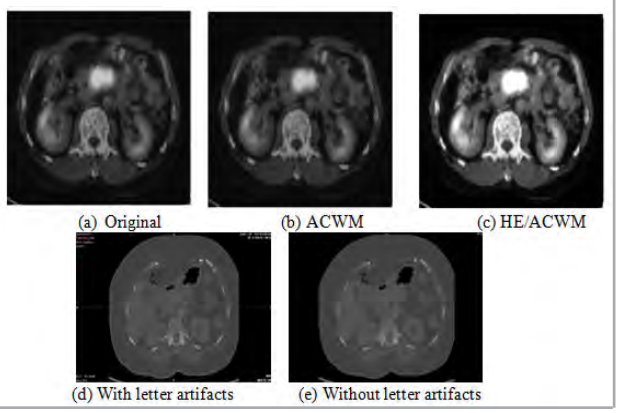
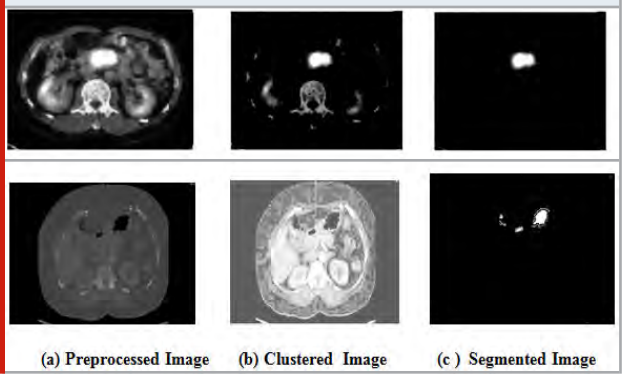


Image segmentation is the partitioning of an image into many components. The goal is to simplify and transform the representation of an image in to something more concrete and easier to analyze. The key goal is to acquire the location of the apprehensive area to aid in identifying and classifying the anomalies as cancerous tissue, benign or malignant. The saliency-based KMC (K-Means Clustering) technique is used for the segmentation in our system. In clustering algorithms, the medical image is commonly over segmented. Salient objects recognition can provide valuable data to enhance the segmentation performance. Segmenting the salient vectors with ' k-means clustering ' is a powerful methodology.

Figure 3: Segmented Images



Saliency technique extents the feature element channels of pixels into the histogram instance to ascertain the spatial difference variation just as assess the saliency of the pixel concerning various pixels in the entire image. In any case, the assessed feature assignments using histogram are discontinuities at the holder edges. Subsequently, the proposed strategy utilizes clustering to dodge the discontinuities at the compartment edges just as in this K--means is used. Below figure demonstrates the segmentation results of pancreatic cancer in PET/CT images (Huang, Zhan and Liang, 2020).

Feature Extraction (FE) is the process involved in analyzing the image texture. The results give a better understanding of texture and object manners determination. When the algorithm has more input data set, it should to be converted in to a smaller dimension for better handling. Converting input image into a normal set of features is termed as FE. By employing FE procedure on the segmented images, pixel group was converted in to a numerical data by the process of feature extraction. The features considered in this effort are mainly GLCM (Grey Level Co-occurrence Matrix) for extracting the statistical features and GRLM (Gray-Level Run-Length Matrix) for extracting the run length features and its procedures are given below.

GLCM method extracts 'texture features' and maintains a relationship among pixels by calculating the 'grey level co-occurrence' values. This method is calculated on the 'conditional probability density' functions 'p (i, j | d,) ' and on selected direction of 'S = 0, 45, 90, 135°', etc., and on distances, d varying from 1 to 5. The function p (i, j | d,) is the probability between two pixels, that are located with an inter sample distance 'd' and a direction 'S', with gray level 'i' and 'j' and this distance is termed as spatial relationship [9]. The significant features of GLCM are Contrast, Correlation, Energy, Entropy and Homogeneity (Sheelakeshvan, Anandan and Balakrishna, 2017).

Table 1. GLCM Features

$\sum_{i,j} \lvert i-j \rvert^2 p(i,j)$	Contrast is the intensity of the given pixel to the entire image.
$\sum_{i,j=1}^{N} P_{i,j}(-\ln P_{i,j})$	Entropy is to measure the randomness's I(x,y) is the probability matrix.
$\sum_{i,j} \frac{(i-\mu i)(j-\mu j)p(i,j)}{\sigma_i \sigma_j}$	Correlation texture calculates the linear dependency of grey levels on those of adjacent pixels.
$\sum_{i,j} \frac{p(i,j)}{1+\lvert i-j \rvert}$	Homogeneity is a measure, which gives the value of how close the components are distributed to that of the diagonal of the matrix.
$\sum_{i,j} \frac{p(i,j)}{1+\lvert i-j \rvert}$	Energy is square of the elements added together in the matrix. Square root of angular second moment.

GRLM is represented in the form of a matrix for geometrical features. It gives a measure of the intensity of the pixels along the given direction mentioned as

Run length. It has two dimensions. Here, each element is represented as the number of components 'j' with the intensity 'i', in the specified directions. In that direction, the 'run-length matrix' estimates for every gray level value how many times the run occur. Whether 2 successive pixels have the same intensity value, next time it takes for 3 pixels and compares it and next it goes for 4 and so on. The length of the run is the number of pixel points in the run (Sindhu and Radha, 2019). Features extracted by GLRM were Where 'Pi'j represents the total number of runs with intensity 'i' also length 'j'. The GLRM features which acquired are utilized for the machine learning algorithms for improving the accuracy in terms of classification as well as detection. Sample features are listed below.

Table 2. GLRM Features

Short Run Emphasis	$\frac{\sum_i \sum_j p(i,j)}{j^2}$
Long Run Emphasis	$\frac{\sum_i \sum_j j^2 p(i,j)}{\sum_i \sum_j p(i,j)}$
Gray Level Non-uniformity	$\frac{\sum_j (\sum_i p(i,j))^2}{\sum_i \sum_j p(i,j)}$
Run Length Non-uniformity	$\frac{\sum_i (\sum_j p(i,j))^2}{\sum_i \sum_j p(i,j)}$
Run Percentage	$\frac{\sum_i \sum_j p(i,j)}{n}$
Low Gray Level Run Emphasis	$\frac{\sum_i \sum_j \frac{p(i,j)}{i^2}}{\sum_i \sum_j p(i,j)}$
High Gray Level Run Emphasis	$\frac{\sum_j \sum_i i^2 p(i,j)}{\sum_i \sum_j p(i,j)}$

Feature selection section deals with the optimizers for an effective feature selection. To discuss the working mechanism of the proposed optimizer, the preliminary background of PSO and whale optimization algorithms are presented here. Feature selection is to be converted in to a more reliable and suitable form for the classifier to classify the cancer cell category. This paper introduced the Whale optimized features for machine learning algorithms to classify the normal and abnormal tumors in PET/CT images of pancreas. Meanwhile, the proposed algorithm has been compared with PSO algorithm in terms of accuracy, precision and recall with the machine-learning algorithm (Sindhu and Radha, 2019).

Particle Swarm Optimization (PSO) is a randomly determined optimization technique copied from herds of birds or else schools of fish. The flock of birds (swarm) has learnt a co-operative method to discover food and every bird in the swarm, changes the hunt model according to their learning knowledge. The concept of PSO algorithm is related to evolutionary algorithm and swarm artificial life systems. The particles in the swarm (Birds) openly fly over the multidimensional search space. Through the trip, every particle creates its individual velocity along with location. By updating of each particle, the entire population is updated (Sindhu and Radha, 2020). The swarm arrangement drives itself, to move toward the point of upper target function value and in the end the particles assemble around this point

The steps of particle swarm optimization are as follows:

$$\vec{V}_i = W\vec{V}_i + c_1 R_1(\vec{P}_{i,best} - \vec{P}_i) + c_2 R_2(\vec{g}_{i,best} - \vec{P}_i)$$

$\vec{V}_i$ = velocity of particle 'i'

$\vec{P}_{i,best}$ = finest position reached by the particle

$\vec{g}_{i,best}$ = best location remembered by the particle individual

'W' = parameter controlling the flying elements

R1, R2 =random numbers among 0 and 1

c1, c2 =cognitive learning factor and social learning factor.

The inclusion of variables of each particle gives the PSO, the facility of correctness in searching. The weighing aspects c1, c2 avoid collision among the particles (individuals). After updating particle, I, velocity v and random number r is verified also protected in a range indicated, to evade collision.

Step 3: Updating of position – There is an interval among succeeding iterations and hence the positions of the particles undergo change as in below equation.

$$\vec{P}_i = \vec{P}_i + \vec{V}_i$$

After refreshing, $\vec{P}_i$ must be verified and in the allowable range.

Step 4: Updating of memory – Update $\vec{P}_{i,best}$ and $\vec{g}_{i,best}$ using the formula as in below equations,

$$\vec{P}_{i,best} = \vec{P}_i \; if \; f(\vec{P}_i) > f(\vec{P}_{i,best})$$

$$\vec{g}_{i,best} = \vec{g}_i \; if \; f(\vec{g}_i) > f(\vec{g}_{i,best})$$

Where $(\vec{x})$ is the point function subject to extension.

Step 5: Destination Checking – The technique iterates steps 2 to 4 until definite end states are reached, for a specified number of iterations, when ended. The estimation of $g_{i,best}$ and $\vec{P}_{i,best}$ give the result.

The fitness values are not considered in PSO algorithms. This is a big computational advantage over other

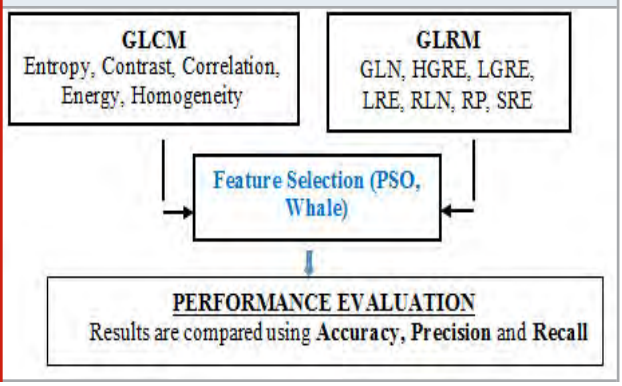
algorithms, when the population is huge. Arithmetic operation of real numbers is used for calculation of velocity and position. The disadvantages as seen in PSO are non-optimal tuning of input features and PSO is one-way information sharing mechanism. In PSO $\vec{g}_{i.best}$ gives information to others (Sindhu and Radha, 2020).

For WHALE Optimization Algorithm (WOA), lately there has been developing enthusiasm for WOA, which was proposed. This hunt and advancement calculation is a scientific reenactment of the conduct and development of humpback whales as they continued looking for food and arrangements. WOA has motivated by the Bubble-net assaulting system, where the whales begin focusing on fish by making winding formed air pockets around their fish down to 12 meters deep from the surface, and afterward, they swim back up to trap and catch their focused-on fish. In light of the general places of whales, in this calculation, the investigation procedure is spoken to by the irregular pursuit of food, which can be scientifically interpreted by refreshing the old arrangements as opposed to picking the best ones through haphazardly choosing different arrangements. Notwithstanding this intriguing conduct, WOA is quite recognized from other improvement calculations, since it just needs to modify two parameters. These parameters make it conceivable to change easily between both the abuse and investigation forms (Sindhu and Radha, 2020).

Table 3. Sample Features (GLCM and GLRM)

GLN	HGRE	LGRE	LRE	RLN	RP	SRE	Entropy	Contrast	Correlation	Energy	Homogeneity
1.88E+02	1.88E+02	18.90997	8.92E+03	19.70096	0.014204	0.202363	0.019676	0.977205	0.439465	0.990878	0.392195
1.73E+02	1.73E+02	24.80303	9.29E+03	22.38788	0.014323	0.230632	0.017115	0.983738	0.399594	0.991884	0.362678
1.69E+02	1.69E+02	43.92917	6.26E+03	54.20417	0.018676	0.34041	0.017303	0.981196	0.425729	0.991839	0.341882
1.43E+02	1.43E+02	45.60367	4.78E+03	1.10E+02	0.023758	0.456583	0.019706	0.981333	0.415898	0.991073	0.393606
1.45E+02	1.45E+02	70.64591	2.09E+03	3.27E+02	0.047098	0.557324	0.007082	0.991682	0.424758	0.996459	0.323678
1.75E+02	1.75E+02	49.05833	5.91E+03	1.22E+02	0.020947	0.458996	0.007059	0.991407	0.433409	0.996471	0.364919
1.57E+02	1.57E+02	68.42647	4.53E+03	1.38E+02	0.026993	0.464515	0.003504	0.99447	0.478787	0.998248	0.240268
1.68E+02	1.68E+02	65.75422	5.37E+03	81.96811	0.021158	0.401881	0.008093	0.990455	0.417807	0.995954	0.261234
8.86E+03	8.86E+03	1.10E+02	8.93E+03	2.82E+02	0.066261	0.087273	0.008063	0.990115	0.426659	0.995968	0.269861
8.09E+03	8.09E+03	1.09E+02	9.94E+03	2.11E+02	0.060265	0.077951	0.007877	0.99094	0.419566	0.996061	0.270785
7.58E+03	7.58E+03	1.07E+02	1.09E+04	1.80E+02	0.056244	0.071496	0.007462	0.991163	0.426729	0.996269	0.278193
7.74E+03	7.74E+03	1.08E+02	1.09E+04	1.97E+02	0.05751	0.070797	0.016464	0.979329	0.441869	0.992265	0.312901
8.06E+03	8.06E+03	1.08E+02	1.02E+04	2.03E+02	0.060005	0.073954	0.011021	0.985145	0.447876	0.994717	0.368355
1.45E+04	1.45E+04	1.18E+02	5.58E+03	1.30E+03	0.110004	0.182276	0.017171	0.976331	0.489616	0.992137	0.297284
8.69E+03	8.69E+03	1.10E+02	9.91E+03	2.51E+02	0.064957	0.076918	0.016008	0.98073	0.428192	0.992723	0.348569
9.65E+03	9.65E+03	1.12E+02	8.64E+03	3.69E+02	0.07238	0.091151	0.023718	0.975637	0.402358	0.988865	0.255592
8.20E+03	8.20E+03	1.09E+02	1.08E+04	2.47E+02	0.061073	0.08131	0.026104	0.975176	0.385267	0.98792	0.247635
8.67E+03	8.67E+03	1.10E+02	9.94E+03	3.10E+02	0.064804	0.094248	0.016256	0.980206	0.443261	0.992374	0.452256
1.20E+04	1.20E+04	1.15E+02	6.79E+03	6.70E+02	0.090691	0.126116	0.015332	0.984463	0.402466	0.992685	0.243327

Figure 4: Feature Selection system



In the following section, we will describe the mathematical model of encircling prey, searching for prey, and spiral bubble-net foraging man oeuvre. For the encircling prey, by the rising total number of iterations from start to an extreme number, humpback whales encircle the prey also update their location in the direction of the finest search agent. We can mathematically formulate this behavior as: If (p<0.5 and mod (U) <1) Then the position of the candidate position X (t+1) is updated in the subsequent equations.

$$D = \text{mod}\ \{(C.X)-X(t)\} \qquad X(t+1) = [X(t) - \{U.D\}]$$

Anywhere p =0.1 (constant) X (t+1) is the best position in the current situation. U and D are calculated by the following equations U= mod {2.a.r-a} C=2.r

Where a is linearly decreases from 2 to 0 and r is the randomly selected vector

In prey searching mechanism, X is replaced with the random variables Xrandom and mathematical equation are given as follows

$$D= \text{mod } \{(C.Xrandom)-X(t)\}$$
$$X(t+1) = [Xrandom\ (t) - \{U.D\}]$$

The encircling the prey and spiral updating of the prey has been done during the exploration phase of whale optimization algorithm. The mathematical expression for updating of new position during the spiral process is given in the below equation.

$$X(t+1) =Dl.ebl.\cos(2\pi l) + X^*(t)$$

Where D is the distance between the new position and updated position in new generation, b is the constant, which varies from 0 to 1.For ML Algorithms for Classification, the majority of classification system uses supervised learning. All the data are labelled and the algorithms learn to predict the output from the training data. This research applies some ML classification techniques such as DT, KNN, SVM then AdaBoost with Ensemble KNN-SVM and demonstrates all classification algorithm's performance on selected features.

Decision tree (DT): DT is a decision care tool, which utilizes a tree like graph of decisions to classify the data.K-Nearest Neighbor (KNN): Which assigns a class based on the most frequent class among the patterns in the neighborhood. Support Vector Machines (SVM): Classifies the data based on the concept of decision planes. AdaBoost with Ensemble KNN-SVM algorithm: In classification algorithms, each one has its own advantage and disadvantage. So, AdaBoost with Ensemble SVM-KNN algorithm is compared with above mentioned algorithms to achieve the highest accuracy then others. The working mechanism of proposed algorithm is explained in the below section. In proposed technique, KNN technique discovers the distance among test sample and training sample.

A significant job of KNN is to catch out the neighbors first and it has been classifying the request sample on the mainstream class of its nearest neighbors. The proposed ensemble (KNN-SVM) classification method can be utilized powerfully for pancreatic cancer classification with less computational complexity in the training as well as detection stage. The lesser computational energy is acquired from KNN method, which does not essential building of a feature space. KNN technique has been utilized in the proposed hybrid approach KNN-SVM as the first step in the pancreatic tumor classification then the SVM technique is established in the 2nd phase as a classification machine of this ensemble model (Sindhu and Radha, 2018).

Adaboost is an iterative approach for improving the classification of the poor classifiers. Algorithm based on Adaboost allocates variant weights to any observation at the primary stage. The weight imposed on the misclassified results will increase after a few iterations, and vice versa, the correctly classified will have fewer weights. The weights on the observations are the measures of the class to which the observation belongs, thereby minimizing the misclassification of the observation while at the same time significantly improving the efficiency of the classifiers. That's mostly aimed at reducing variance, boosting is a technique consisting of fitting sequentially multiple poor learners in a very adaptive manner, each model in the sequence is equipped to give more importance to observations in the dataset that were treated badly in the sequence by previous model (Siqi and Hyian, 2019).

Algorithm: AdaBoost with Ensemble KNN - SVM classifier

Input: PET/CT Pancreatic images with class label (benign or malignant) i.e. (X1, C1),(X2, C2)... (Xn, Cn);

Feature pool F= {fm, m=1... n}; Number of iterations = R

Initialization: Weight of each features

$$\frac{1}{N}; \forall\ i\ (i= 1,\ldots, N); \sigma = 1000$$

For r = 1 to R do:
(a) Generate a training set by sampling with {wi(r)}
(b) Train base classifier hr ((Proposed Ensemble Classifier)) by this training set
1. Apply SVM classifier on PET/CT data set with K-fold cross-validation and K=10.
2. Update the weights.
3. According to Wolfe dual form, weight minimization is

$$Minimize: w(\alpha) = -\sum_{i=1}^{N} \alpha_i + \frac{1}{2}\sum_{i=1}^{N}\sum_{j=1}^{N} y_i y_j \alpha_i \alpha_j k(X_i, X_j)$$

$$Subject - to: \sum_{i=1}^{N} y_i \alpha_i = 0, \forall_i; 0 \le \alpha_i \le C$$

4. Predict the test PET/CT class using the cross validated model with minimum weight.
5. Develop weighted KNN Classifier with number of nearest neighbors K=10 on PET/CT data set.
6. Apply K-fold cross validation with K=10.
7. Weight contribution of each k neighbor
8. Set initial weights of KNN = updated minimum weights of SVM.
9. Xt is test PET/CT image

$$\hat{f}(X_t) \leftarrow \frac{\sum_{i=1}^{k} w_i f(X_i)}{\sum_{i=1}^{k} w_i}$$

10. Predict the test PET/CT class using the cross validated model with minimum weight.
11. Take weighted average of predictions from both the models.
(c) Compute the training error of hr:

$$\epsilon_r = \sum_{i=1}^{N} w_i(r).I[C_i \neq h_r(X_i)]$$

In the above equation, I ∈ (-1, 1), IA is indicator of A; we assume (∈_r<0.5)
Set:

$$\alpha_r = log(\frac{1-\epsilon_r}{\epsilon_r})$$

(We have αr > 0) Update the weights by:

Output

$$w_i'(r+1) = w_i(r)exp(\alpha_r I[C_i \neq h_r(X_i)])$$

$$w_i(r+1) = \frac{w_i'(r+1)}{\sum_i w_i'(r+1)}$$

$$h(x) = Sign(\sum_{r=1}^{R} \alpha_r h_r(X))$$

AdaBoost with ensemble KNN-SVM as component classifier for pancreatic cancer classification. Proposed scheme gives classification accuracy of 98.3% for pancreatic PET/CT classification. Results reveal that proposed AdaBoost with ensemble KNN-SVM outperforms other methods.

RESULTS AND DISCUSSION

In order to test and assess the proposed system, totally 119 PET/CT pancreatic images of 50 to 71-year-old patients confined from 2016 to 2018 are taken as an input images, going to the two types such as benign and then malignant (Sindhu and Radha, 2019). Performance Evaluation: The PSO and WAO features, which are extracted from the datasets, are utilized for training as well as testing system. For valuation, 80% of data were taken for training and then 20% of data were taken for testing. The assessment is accepted for the different algorithms with the below parameters.

$$\text{Accuracy} = \frac{DR}{TNI} \text{ x100}$$

$$\text{Precision} = \frac{TP}{TP+FP} \text{ x100}$$

$$\text{Recall} = \frac{TN}{TP+FN} \text{x100}$$

Where TP, TN, FP and FN denote 'True Positive', 'True Negative', 'False Positive' and 'False Negative' values and 'DR' and 'TNI' Represents total Number of 'Detected Results' and 'Total number of Iterations' [10]. The performance of the proposed algorithms has been assessed by various cases which are shown in the below table, In the evaluation scenario, pancreatic tumor is considered for the classification and different comparative analysis are shown in above table.

Accuracy analysis: The below figure clearly shows accuracy of the Whale based optimization has a maximum accuracy when compared with the other techniques.

Precision and Recall analysis: Again, the precision and recall has been calculated and compared with the other algorithms in which the whale-based technique outperforms the other algorithms.

Figure 5: Encircling Attack Prey Searching Methodology for Hump Back Whales

Table 4. Comparison of Optimization Techniques with ML

Description	Algorithm used	Accuracy	Precision	Recall
Machine Learning Algorithms	SVM	80.7%	92	86
	DT	89.9%	90	78
	KNN	90.8%	96	92
	Adaboost ensemble	95.8%	96	93
Optimization Technique	PSO-Adaboost ensemble	96.6%	94	100
	Whale-Adaboost ensemble	98.3%	97	100

The above diagram clearly shows that whale optimization technique based on Ensemble algorithm gives the best results when compared with others. The feature selection and ML algorithm provided by the MATLAB machine-learning toolbox is utilized for assessing the effort of the proposed methodology and calculates the number of normal and abnormal cancer present in the testing dataset. 119 data were taken for analysis. Among that, 20% has been taken as testing data and the 80% has been

Figure 6: (a) Comparative analysis with Accuracy

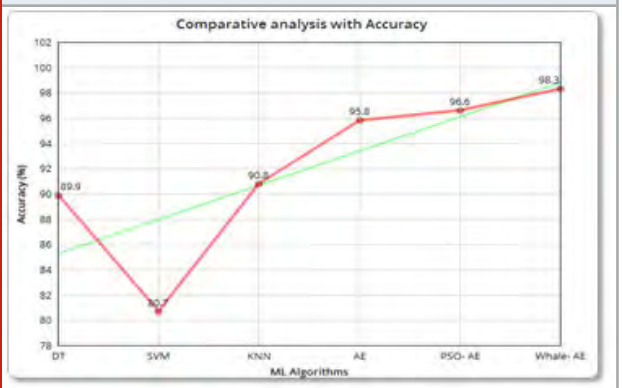


Figure 7: (b) Comparative analysis with Precision

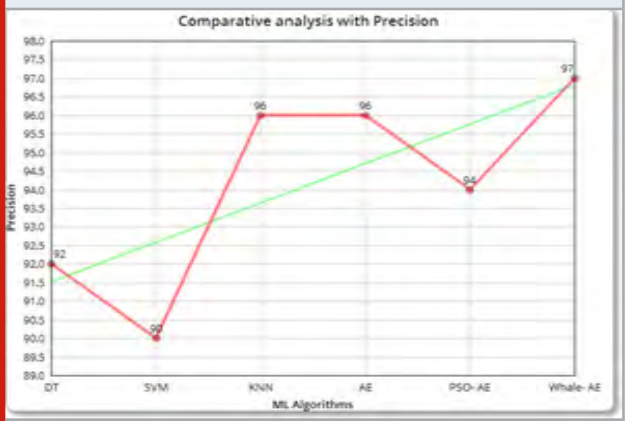


Figure 8: (c) Comparative analysis with Recall

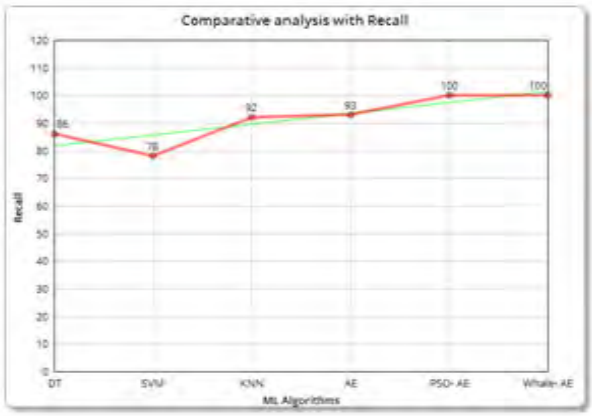


Figure 9: Confusion Matrix Whale-Adaboost Ensemble

Table 5. Comparison of Previous Work

Author	Year	Pre-processing	Segmentation	Feature Extraction and Selection	Classification	Accuracy
Jeenal Shah et al.,	2015	Low Pass filter (Non-Linear filter)	-	-	Minimum distance Classifier	65.26%
Siqi Li et al.,	2019	-	Simple Linear iterative clustering	PCA(Principal Component Analysis)	HFB-SVM-RF	96.47%
Proposed Methodology	2020	HE/ACWM	Saliency based K-Means Clustering	WOA(Whale Optimization Algorithm)	Adaboost ensemble KNN-SVM	98.3%

taken as training data. With this the accuracy percentage of Whale-Adaboost ensemble algorithm has been reached with 98.3%. According to that, 1.7% of data comes under misclassification scenario.

Whale-Adaboost ensemble algorithm reached the maximum accuracy when compared with other algorithms. According to the confusion matrix, 49 cases has been classified under normal and 68 cases has been classified under ab-normal. The remaining two (misclassification) cases may be normal or ab-normal cases (Sindhu and Radha, 2020).

Many imaging modalities are used for the diagnosis of pancreatic cancer. Jeenal Shah et al., utilized a minimum

distance classifier to detect the pancreatic cancer in PET/CT image and they found out to be 65.26% accuracy at the year of 2015[4]. Then, Siqi Li et al., designed a hybrid feedback-support vector machine-random forest (HFB-SVM-RF) model to identify normal pancreas or pancreas cancer and they achieve 96.47% accuracy in the year of 2019[12]. Compared to both systems, our proposed model obtained 98.3% accuracy with the Adaboost ensemble KNN-SVM technique for pancreas tumour classification (Udhav and Deshmukh, 2018).

CONCLUSION

This research paper proposes a novel CAD system for pancreas cancer on PET/CT scan images, comprising pre-processing, pancreatic segmentation, feature Extraction, selection, and classification respectively. Noise and artifact removal is performed using the filters, the segmentation is performed using the novel method of saliency, and features are extracted using GLCM and GLRM, then a feature selection is applied based on WAO and the finally with regards to WAO – Adaboost ensemble KNN-SVM algorithm, for detecting and classifying the pancreatic cancer. We perform the identification task for 119 PET/CT images. The implementation outcomes and evaluations with the related work demonstrates that our proposed system can reach better classification performance than others system.

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Barriers Preventing the Society from Seeking Mental Health Services: A Cross-Sectional Saudi Arabian Study

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ABSTRACT

The objective of the present study was to investigate the misconceptions Saudis hold regarding psychiatric disorders, people with such disorders, and psychiatric care. A cross sectional study was done in Saudi Arabia during 2019 using surveys, where 1,170 completed questionnaires were received, and data was analyzed using the $\chi 2$ test to determine whether responses to certain questionnaire items varied with respondents' ages and genders. We selected $p < 0.05$ as our statistical significance threshold. We found that the prevalent misconceptions included beliefs that psychiatric care is expensive and that mental hospitals are old. The respondents' ages were not associated with the likelihood of believing that psychiatric care is expensive or that ill persons should be isolated from the public. However, we found that women were more likely than men to express definite opinions on certain statements on the questionnaire. Our findings suggest that stigmatization against persons with psychiatric disorders may not be a major factor preventing Saudis from seeking psychiatric care. Moreover, positive attitudes towards traditional methods and spiritual mediators were not as common as we had expected. We encourage future studies in Saudi Arabia about the approaches to treating psychiatric disorders and the need for psychiatric care and services awareness among the population.

KEY WORDS: SAUDI ARABIA, PSYCHIATRY, MENTAL HEALTH, HELP-SEEKING, MISCONCEPTIONS, STIGMA.

INTRODUCTION

Belief is a definite feeling that something exists or that a claim is true, and misconceptions are incorrect ideas based on misinterpreting situations. Beliefs and misconceptions differ widely between cultures and individuals (Dardas and Simmons 2015). Misconceptions about psychiatric disorders are widespread in many cultures. This is problematic because such misconceptions about psychiatric disorders may cause people with psychiatric disorders to be unwilling to seek appropriate care. In the case of Saudi Arabia, mental health is an issue of growing importance (Elbur et al. 2014), but its taboo nature in Saudi culture has adversely affected the willingness of people in Saudi Arabia to seek psychiatric care. Abolfotouh et al. (2019) have recently shown the lack of knowledge and stigmatizing attitude contributed to Saudis toward psychiatry consultation. Recently, Mahsoon et al. (2020) have also demonstrated that patients with psychiatric illnesses regardless of the positive view to help seeking and family support have significant prejudices about mental health in Saudi Arabia, (Mahsoon et al. 2020).

Several studies have investigated stigmas around psychiatric disorders in various cultures. For example,

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Abdullah and Brown (2011), who conducted such an investigation by analyzing the beliefs and values of different cultural groups, reported that people of Middle Eastern descent usually value family honor, which is grounded in maintaining good appearances and reputations, concealing emotions, and respecting patriarchal norms. Abdullah and Brown concluded that these values contribute to the stigma that generally surrounds psychiatric disorders in Middle Eastern cultures. Similarly, Al-Adawi et al. (2002) reported that the attitudes of Omanis towards psychiatric disorders are mainly affected not by demographic variables but rather by the degree to which traditional beliefs are accepted.

A study that investigated attendance at a mental health facility in Riyadh found that the factors contributing to nonattendance included, but were not limited to, negative beliefs about psychiatric disorders in general, the standard ways of treating psychiatric disorders in the community, and the perceived ineffectiveness of available mental health treatments (Alnamlah 2006). The reluctance to seek medical advice and to admit the presence of a psychiatric disorder is found in a study done among emirates by Eapen and Ghubash (2004). Furthermore, Soheilian and Inman (2009) found that most Americans of Middle Eastern descent would avoid psychiatric counseling for the sake of avoiding damage to their families' reputations and images.

Similar findings have been reported by studies focused on non–Middle Eastern populations. For example, Henderson et al. (2013) analyzed data from England and identified multiple barriers that can prevent an individual from seeking psychiatric help, such as the inability to identify psychiatric disorders, ignorance concerning how to access psychiatric services, and preconceptions and assumptions that psychiatric patients will be unfairly treated by society. Similarly, Corrigan et al. (2014) reviewed studies from around the world and reported that stigmas around psychiatric disorders can prevent an affected individual from pursuing psychiatric care. Moreover, the available literature on the utilization of psychiatric care in military and veteran populations indicates that beliefs and misconceptions concerning mental health contribute to individuals being unwilling to access psychiatric services (Vogt 2011). Individuals with psychiatric disorders who are unemployed commonly encounter various barriers when attempting to obtain psychiatric care, such as discrimination and a lack of knowledge concerning mental health and the conditions of psychiatric services (Staiger et al. 2017).

Gulliver et al. (2010) reported that young adults and adolescents can encounter similar obstacles, and Jang et al. (2011) showed that older individuals frequently have negative perceptions of mental health services, with many believing that having psychiatric issues would disappoint and upset not just the affected person but also the affected person's family. Most of the available studies concerning attitudes towards psychiatric disorders have focused on cultures, groups, and countries outside the Middle East, and few studies have been conducted in Saudi Arabia. We, therefore, investigated the beliefs and misconceptions that Saudis hold about psychiatric disorders, people with such disorders, and psychiatric care. We hope that elucidating such beliefs and misconceptions will facilitate the development of appropriate interventions to counteract their harmful effects.

MATERIAL AND METHODS

The aim of this study is to find what keeps Saudis population from seeking psychiatric help. Where we conducted a cross-sectional survey of residents of Jeddah, Saudi Arabia, between January 2019 and May 2019. We randomly distributed electronic and paper surveys based on a modified version of the Community Attitude towards the Mentally Ill (CAMI) scale in multiple settings and accepted responses from any Saudi who was willing to participate. We translated the CAMI questionnaire to Arabic language and then rechecked it to make CAMI culturally appropriate for Saudi. The responses were collected from 1,424 individuals, of whom 1,170 completed the CAMI questionnaire and were included in our analyses. Thus, the response rate was 82.1%. The collected data were analyzed with SPSS software version 26 (IBM, Armonk, NY, USA). These analyses included the use of the $\chi 2$ test to determine whether responses to certain questionnaire items varied with respondents' ages and genders. We selected $p < 0.05$ as our statistical significance threshold.

Table 1. Demographic characteristics of the respondent sample (n = 1,170)

Variable	No. (%) of respondents in the category
Age, y	
15–24	622 (53.2%)
25–34	221 (18.9%)
35–44	204 (17.4%)
45–54	94 (8.0%)
≥55	29 (2.5%)
Gender	
Male	238 (20.3%)
Female	932 (79.7%)
Education level	
Basic education	271 (23.2%)
Technical education	82 (7.0%)
University	747 (63.8%)
Postgraduate studies	70 (6.0%)

RESULTS AND DISCUSSION

Of the 1,170 individuals whose survey data were included in our analyses, 53.2% were younger than 25 years and 79.7% were female (Table 1). The study sample is representative of the Saudi population as the median age of Saudi Arabia is 30 years according to the statistical

yearbook done in 2018 by the General Authority of Statistics.

Many of the respondents disagreed with most of the questionnaire statements listed in (Table 2), which generally expressed negative attitudes towards persons with psychiatric disorders, mental health facilities and services, and expenditures on psychiatric care. The questionnaire also included some statements that expressed support for using traditional methods, such as spiritual mediators and folk medicine, to treat psychiatric disorders.

Table 2. Responses concerning psychiatric disorders and services, a psychiatric patient and their integration into the community

Statement	No. (%) of respondents who agreed	No. (%) of respondents who disagreed
"Mental patients should be encouraged to assume the responsibilities of normal life."	1,060 (90.6%)	24 (2.1%)
"I would not want to live next door to someone who has been mentally ill."	76 (6.5%)	844 (72.1%)
"The mentally ill should be isolated from the rest of the community."	19 (1.6%)	656 (56.1%)
"The mentally ill are a burden on society."	78 (6.7%)	948 (81.0%)
"A woman/man would be foolish to marry someone who has suffered from mental illness, even though they seem fully recovered."	162 (13.8%)	805 (68.8%)
"Less emphasis should be placed on protecting the public from the mentally ill."	70 (6.0%)	294 (25.1%)
"The best way to handle the mentally ill is to keep them behind locked doors."	22 (1.9%)	1,082 (92.5%)
"Anyone with a history of mental problems should be excluded from public places."	61 (5.2%)	957 (81.8%)
"Mental hospitals are an outdated means of treating the mentally ill."	655 (56%)	146 (12.5%)
"The mentally ill do not deserve our sympathy."	28 (2.4%)	1,062 (90.8%)
"Mental health facilities should be kept out of residential neighborhoods."	126 (10.7%)	778 (66.5%)
"The mentally ill should not be given any responsibility."	104 (8.9%)	785 (67.1%)
"It is best to avoid anyone who has mental problems."	95 (8.1%)	836 (71.5%)
"Mental health care is expensive."	262 (22.3%)	110 (9.4%)
"Increased spending on mental health services is a waste of money."	39 (3.4%)	1,071 (91.5%)
"Mentally ill persons can be treated by spiritual mediators."	241 (20.6%)	494 (42.2%)
"Mentally ill persons can be treated by traditional methods 'folk remedies.'"	283 (24.2%)	344 (29.4%)
"Medications for mental illnesses affect the personalities of patients and make them insensible."	438 (37.4%)	244 (20.9%)
"Medications for mental illness cause addiction."	986 (84.2%)	33 (2.8%)

Although 90% of respondents believed that psychiatric patients should assume normal responsibilities in society, 37.4% agreed that psychiatric medications affect patients' personalities and make them insensible. Moreover, 84% thought that psychiatric medications can be addictive. When asked about their perceptions of mental hospitals, 50.6% agreed that they are an outdated means of treating psychiatric disorders. Furthermore, most respondents agreed that psychiatric care is expensive. When we tested for relationships between the respondents' ages and their attitudes towards mental health, we observed no significant differences in the levels of agreement with most statements (Table 3).

However, individuals who were 35 years old or younger were more likely than older respondents to agree that mental hospitals are outdated as treatment settings ($p < 0.001$). Furthermore, when we tested for relationships between the respondents' genders and their attitudes towards mental health, we found gender-based differences in responses (Table 4).

Relative to male respondents, female respondents were less likely to be neutral on the questions of whether mental health care is expensive ($p = 0.001$) and whether psychiatric patients should be isolated from the community ($p = 0.04$). Female respondents were also more likely to agree that mental hospitals are outdated as treatment settings ($p = 0.01$). In this study, we did not find evidence of negative attitudes towards persons with psychiatric disorders being common among Saudis. Furthermore, we did not find strong evidence of sociodemographic factors being relevant to such beliefs and misconceptions among Saudis, although we did observe some gender-based differences in beliefs concerning psychiatric care and whether psychiatric patients should be isolated from the community.

Some people may avoid seeking help because their relatives harbor unfavorable attitudes towards psychiatric patients (Al-Adawi et al. 2002). However, we did not find evidence of such attitudes being common among Saudis. In fact, we found that many survey respondents favored integrating persons with psychiatric disorders into society, did not believe that protecting the public from such persons should be emphasized, and did not believe that increased public expenditures on mental health services would be a waste of money. Most of our respondents encouraged persons with psychiatric disorders to assume normal responsibilities in society.

Table 3. Attitudes towards persons with psychiatric disorders and psychiatric care according to age group

Statement and possible responses	No. (%) of respondents aged ≤35 years (n = 843) providing a given response	No. (%) of respondents ages >35 years (n = 327) providing a given response	χ2 test result for between -group difference in responses	p Value
"Mental health care is expensive."				
Agree	431 (51.1%)	154 (47.1%)		
Disagree	84 (10.0%)	31 (9.5%)	2.01	0.3
Neutral	328 (38.9%)	142 (43.4%)		
"The mentally ill should be isolated from the rest of the community."				
Agree	43 (5.1%)	10 (3.1%)		
Disagree	480 (56.9%)	183 (56.0%)	2.7	0.2
Neutral	320 (38.0%)	134 (41.0%)		
"Mental hospitals are an outdated means of treating the mentally ill."				
Agree	477 (56.6%)	178 (54.4%)		
Disagree	123 (14.6%)	23 (7.0%)	18.02	<0.001
Neutral	243 (28.8%)	126 (38.5%)		
"Medications for mental illnesses affect the personalities of patients and make them insensible."				
Agree	312 (37.0%)	126 (38.5%)		
Disagree	182 (21.6%)	62 (19%)	0.9	0.6
Neutral	349 (41.4%)	139 (42.5%)		

This last finding is similar to the finding of Al-Adawi et al. (2002) that most of their Omani respondents believed that patients with psychiatric disorders can be treated and should be considered members of the community. Futhermore, discrimination against psychiatric patients remains a barrier that prevents some people from seeking help (Gulliver et al. 2010). Where a study by (AlAteeq et al. 2018) concluded that family participation in the care plan for a patient and cultural differences had a big impact in developing self-stigmatization. Moreover, keeping a patient privacy and over protecting it is another explanation of the discrimination facing psychiatric patients, mental health care and its services. The negative attitude of young adults investigated by Mahsoon et al. (2020) toward patients with psychiatric illnesses regardless the positive view to help seeking and family support shows the significant prejudice about mental health in Saudi Arabia.

Our survey respondents generally agreed with statements describing psychiatric hospitals as outdated institutes. However, Jang et al. (2011), who surveyed Hispanic older adults in the US, found that their respondents generally had positive attitudes towards mental health services, although this did not extend to psychiatric counseling specifically. The mental health system in Saudi Arabia has been improving by the support of the Ministry of Health, where Primary health care incorporated outpatient, inpatient, residential facilities and mental health hospitals dedicated toward psychiatric patients (Qureshi et al. 2013).

A study of attendees at a mental health facility in Riyadh found that many respondents held negative attitudes towards psychotropic medications, believing that such drugs are ineffective or only become effective after they have been taken for a long time (Alnamlah 2006).

Gulliver et al. (2010) found that a lack of knowledge is a potential barrier preventing young people from accessing mental health services. The same authors also found that misconceptions concerning psychiatric medications, such as the belief that such drugs are addictive or can affect patients' personalities, were also potential barriers. Abolfotouh et al.(2019) have recently shown the lack of knowledge and stigmatizing attitude contributed to Saudis toward psychiatry consultation.

Table 4. Attitudes towards persons with psychiatric disorders and psychiatric care according to gender

Statement and possible responses	No. (%) of men (n = 238) providing a given response	No. (%) of women (n = 932) providing a given response	χ2 test result for between-group difference in responses	p Value
"Mental health care is expensive."				
Agree	100 (42.0%)	485 (52.0%)		
Disagree	17 (7.1%)	98 (10.5%)	14.4	0.001
Neutral	121 (50.9%)	349 (37.4%)		
"The mentally ill should be isolated from the rest of the community."				
Agree	8 (3.4%)	45 (4.8%)		
Disagree	121 (50.8%)	542 (58.2%)	6.4	0.04
Neutral	109 (45.8%)	345 (37.0%)		
"Mental hospitals are an outdated means of treating the mentally ill."				
Agree	120 (50.4%)	535 (57.4%)		
Disagree	43 (18.1%)	103 (11.1%)	9.1	0.01
Neutral	75 (31.5%)	294 (31.5%)		
"Medications for mental illnesses affect the personalities of patients and make them insensible."				
Agree	91 (38.2%)	347 (37.2%)		
Disagree	52 (21.8%)	192 (20.6%)	0.4	0.8
Neutral	95 (39.9%)	393 (42.2%)		

In this study, we did not find a significant difference between the use of traditional and psychiatric methods among Saudis. This is despite the fact that traditional approaches are not clinicians' first choices for treating psychiatric disorders. Notably, the aforementioned study from Riyadh found that belief in traditional healing methods was a potential reason for not seeking psychiatric care, with many members of the Saudi public attributing psychiatric disorders to demonic influences (Alnamlah 2006).

We encourage future studies in Saudi Arabia about the approaches to treating psychiatric disorders and the need for psychiatric care, health services awareness and the use of technologies among the population in treating the patients.This study's major limitation was the manual distribution of questionnaires, which made it difficult to obtain fully answered surveys. Another limitation was the overrepresentation of certain groups such as female responders more than male and university graduates in general.

CONCLUSION

We investigated the beliefs and misconceptions that may prevent Saudis from seeking psychiatric evaluations, and we found that many Saudis hold the belief that psychiatric treatment is expensive and that mental hospitals are outdated. Moreover, our findings suggest that the stigmatization of psychiatric disorders and discrimination against persons with psychiatric disorders may not be major factors preventing Saudis from seeking psychiatric care. Furthermore, positive attitudes towards traditional methods and spiritual mediators were not as common as we had expected.

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Sacred Groves and Honey Bee Conservation in Rural Villages of Bankura District, West Bengal, India

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ABSTRACT

Sacred groves are virgin forest patches with rich diversity, conserved by the local people for centuries with their religious and socio-cultural practices. It connects the past and present community in terms of cultural and religious beliefs and taboos that the deities reside in the sacred groves that protects them from different calamities. Sacred groves can play a vital role in biodiversity conservation because these sites are undisturbed by people. Sacred groves are found in different parts of the world where ethnic people live. In India, sacred groves are more common in the Western Ghats, Northeast India, and Central India, etc, particularly where the indigenous communities live. Several plants and animals are well conserved in these sacred groves. In recent days, due to deforestation and rapid urbanization number of the trees and associated fauna species is decreasing rapidly. In this regard, the present scientific study was carried out in two blocks of Bankura district in West Bengal to document the sacred groves, and its role in biodiversity conservation through its legends, lore, and myths. In these studies, six (6) sacred groves were documented from five (5) villages. It has been noticed that honeybee prepare hives in these sacred trees. They show preference in habitat selection by selecting sacred trees as secure habitat for hive formation and neglecting other trees present surrounding sacred trees. Many other animals are also protected in these sacred groves; Honeybees are also getting direct protection through traditional rituals and religious taboos from poachers and chasers. So, conservation of these sacred trees and traditional thoughts is needed for the conservation of local fauna. But more research on the specific site selection of sacred trees by honey bees for hive formation is needed for understanding their behaviour and successful conservation.

KEY WORDS: SACRED GROVES, COMMUNITY, BIODIVERSITY, HABITAT, RITUALS, BEHAVIOUR, CONSERVATION..

INTRODUCTION

Sacred groves (SGs) are a small fragment of nature saturated with diverse vegetation, devoted to the local deities, and considered by local people as divinity proprietary. Sacred groves and sacred trees are protected, unmolested, and conserved for centuries by the local communities by performing religious rubric, customs, and traditional rituals. Human beings and nature are connected deeply through these traditional worship practices. In many parts of the world, various sacred groves (SGs) are observed where local people worship many spiritual activities. These type of SGs are commonly observed in many countries like China, Srilanka, Bangladesh, Nigeria, Mexico, Syria, Ghana and it is very common in North-Eastern states of India like Manipur, Mizoram, Arunachal Pradesh, and also in Bihar, Jharkhand, Orissa, Madhya Pradesh, Andhra Pradesh, Karnataka, Tamil Nadu, West Bengal, and other states. In Maharashtra it is locally known as Devrai and Deviahate, in central India, these are known as Sarana, Orance in Rajasthan, Kavu, or Nagavanam in Kerala,

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Sidharavanam in Karnataka, Nandavana in Tamil Nadu, and Kavu in Andhra Pradesh. In different parts of West Bengal, these SGs are named according to the names of God and Goddess worshipping in that particular area. Bankura is a traditionally wealthy and culturally opulent district of West Bengal and the witness of an enormous number of sacred groves and sacred trees, preserving nature through its diverse traditions and customs (Mitra et al., 2015).

Sacred groves also provide habitat and protection for many ecologically valuable keystone species, which contribute to the maintenance and enhancement of biodiversity in many parts of the world. SGs, also act as nursery and storehouses of tribal and folk medicinal plants. Valuable medicinal plants like *Azadirachta indica*, *Holarrhena antidysenterica Hemidesmus indicus*, *Aegle marmelos*, and *Leucas aspera* are commonly found in various sacred groves. Deb and Malhotra (1997) did the pioneer research activity on sacred groves of West Bengal. In the Bankura district, from 26 sacred groves, 114 species of flowering plants belong to 52 different families were documented by Basu (2009). Among three community development blocks of Bankura district namely Barjora, Chhatna and Saltora total of 51 economically important plant species were reported by Mitra et al., (2015). Dutta and Mondal (2018) reported the scientific study related to social customs and its role in Bat conservation at Majdiha village of Bankura District. In West Bengal and other parts of India, local people put some symbols like stones, clay deities of horse, elephants and other animal model, pictures, natural symbols like a snakehead, symbol of lotus and many spiritual signs under any big tree and shows respect of their faith upon nature or God or respect to their ancestors (Dutta and Mondal, 2018).

Sometimes ropes are knotted surrounding the trees and usually, local peoples do not cut the trees, enter these sacred zones silently without eating. These groves are undisturbed by villagers even they do not climb on the trees, even not cut the dead woods, green or brown branches. So, in these places, many animals reside especially birds, small reptiles, mammals, and many insects and they are protected because of faith in nature or God of people of that area. Honeybees are one of the best and most important pollinators in the world and honey-producing and wax producing insects. Near about 16% of flowering plants in the world and 400 agricultural plants are pollinated by honeybees (Crane and Walker, 1984). Michener (2007) reported that 17533 species can be found throughout the globe which is categorized under 443 genera and seven families but of them, only 8 species with 43 subspecies are considered as honeybees. Honeybees come under the genus Apis belonging to the family Apidae under the order Hymenoptera and class Insecta (Engel, 1999). In India, five species of honeybees are commonly found, viz. *Apis dorsata* (Rock bee), *Apis laboriosa, Apis cerana* (Indian bee), *Apis florae* (Little bee), and *Apis adereniformis*. *Apis mellifera* (European bee) is not native to India and has been introduced from outside (Jack et al., 2016).

Apis dorsata famous by the name "Rock bee" or "Giant honeybee" has three subspecies, *Apis dorsata* dorsata, *Apis dorsata* breviligula, and *Apis dorsata* binghami. A single *Apis dorsata* colony composes of one queen bee, few drones, and thousands of worker bees. Generally, the Nests of *Apis dorsata* can be found under the branches of large and tall trees, and every single comb nearly 150 cm in length and 70 cm in height (Jack et al., 2016). Preferable trees of *Apis dorsata* for making nests are *Mangifera indica, Terminalia* sp., *Diospyros meloxylon, Persea macrantha, ficus sp., Pterygota alata*, etc. In many parts of the world sacred groves also helps to replenish water resources, improve soil quality and is pivotal for biodiversity conservation of plants and animals including rare, endemic, threatened, vulnerable species and ethno botanical species (Sharma 2020).

Similarly, it provides natural habitat for many endemic, rare, primitive, and economically valuable plants along with a good number of wild animals, amphibians, reptiles, birds, variety of butterflies and insects (Samati and Gogoi, 2007; Sharma 2020). This paper aims at documenting the rituals observed in two blocks of Bankura district and scientific observation have focused on how traditional regulations and religious rituals protecting and conserving various animals especially the honeybees (*Apis dorsata*) in this region, and the selection specificity of honeybees (*Apis dorsata*) toward sacred trees for their hive formation.

MATERIAL AND METHODS

A total of six major SGs trees were surveyed in five villages of Taldangra block and the Onda block of the Bankura district. For collecting primary data, a set of questionnaires were prepared. Usually, elderly people of the surrounding village were selected and interviewed with standard set questionnaires like the name of sacred groves, age of sacred groves, rituals performed near-sacred groves, their perception about the sacred groves, types of animals normally seen near-sacred groves, honey bee hive formation information, etc. Also counted the number of the active beehives and rejected beehives in those sacred trees.

RESULTS AND DISCUSSION

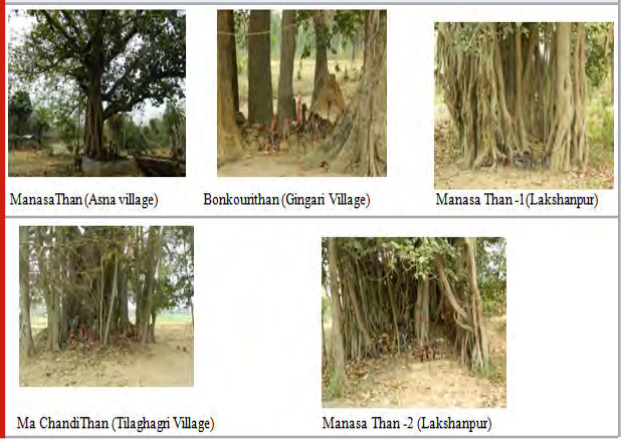
During the present study, a total of six (6) sacred groves (Table 1) are observed in the study area. Plants that are commonly conserved in these sacred groves due to the religious belief are *Ficus benghalensis, Madhuca longifolia*, and *Shorea robusta*. Plant diversity of an ecosystem plays an important role in conserving local fauna. These sacred groves have a great influence on the local flora and fauna of the region as well as the microclimate of that locality. Many wild animals and domestic animals reside there freely. So, indirectly sacred groves conserve local flora and fauna based on their sacred faith (Devakumar et al., 2018). In different parts of the world, sacred groves are found in various forms, including burial grounds, cremation grounds, and sites of ancestral or deity worship (Bhagwat and Rutte,2006).

Various types of myths and faiths are associated with these groves and villagers strictly respect the dignity of that place. The types of rituals that are performed in the sacred groves vary in a different community and also vary from region to region. In these villages, the most sacred groves are dedicated to worshiping "Manasadevi", the goddess of snakes.

Figure 1: Sacred tree with active beehives in Korakanali village, Bankura

These sacred groves are called "Manasa than" and people put clay deities of a horse, elephants under the trees. Similar types of sacred groves are commonly observed in Southern Kerala where sacred groves are dedicated to Naga (Sarpa God). In Northern Kerala, the

Figure 2: Other sacred groves in the study area



groves are named on the presiding deities and locally known as Kotta in Malayalam and are different forms of the Mother Goddess (Kali), Vaishnava, or Shivaite forms. Panda et al. (2003) also reported 10 important sacred groves of Santhals from the Bankura district of West Bengal (Sharma and Kumar, 2020). Honeybees are economically highly important and profitable insects and are also responsible for valuable ecosystem services like pollination. Gallai and Vaissiere (2009) reported that, the total economic value of pollination through honeybees to agricultural production was estimated at about € 153 billion, which was 9.5% of the value of total agricultural production worldwide used for human food for the year 2005.

Table 1. Beehives conserved in sacred grove trees

Sl No.	Name of the SGs and Place	Rituals celebrated	The main plant conserved in SGs	Active Bee hives	Rejected Beehives
1.	Manasa Than Korakanli Village	Every year in the month of mid- August local people worship "Manasadevi", goddess of snakes with clay deities of the horse, elephants, etc under it.	*Ficus benghalensis*	19	28
2.	Ma Chandi Than Tilaghagri Village	Every year during "Aswinsankranti" surrounding these trees people worship Goddess Chandi"	*Ficus benghalensis*	11	9
3.	Manasa Than Asna Village	Villagers worshipping "Manasadevi", goddess of snakes under this tree.	*Ficus benghalensis*, *Madhuca longifolia*	8	14
4	Manasa Than -1 Lakshanpur Village	Under the trees, villagers put clay deities of horse, elephants and, worship "Manasadevi", goddess of snakes.	*Ficus benghalensis*	5	8
5	Manasa Than: -2 Lakshanpur Village	Every day villagers worshipping "Manasadevi", goddess of snakes	*Ficus benghalensis*	6	11
6	Bonkouri than Gingari Village	Every year in the month of mid- January a local large fair is organized during "Ekhansankranti" surrounding these trees and worship clay deities of the horse, elephants, etc under it.	*Shorea robusta*, *Madhuca longifolia*	7	10

Apart from honey pollination and honey production, honeybees are also involved in making other goods like bee wax, pollen, propolis, bee venom, etc (Sharma and Kumar, 2020). But in recent times, due to various human activities like anthropogenic climate impacts, habitat destruction, deforestation, industrialization, rapid urbanization and environmental pollution the variety, abundance, and health of wild pollinators are under threat (Yang et al., 2018).

Underneath the branches of these sacred trees many colonies of *Apis dorsata* have been identified and these beehives are present over there for many years without being disturbed and hunted by any chaser and common people. In Korakanali village SGs maximum of 19 active bee hives observed and also in other sacred groves active hives are seen. *Apis dorsata* specially selects those trees under which rituals were performed and neglect big trees near SGs for hive preparation. The spiritual belief of local communities on these sacred trees giving direct protection to many animals, birds, honeybees, and other organisms. Villagers follow strict rules inside the groves throughout the world. They not cut sacred trees and kill animals inside the groves believing the trees to be the abode of god and ancestral spirits. This gives better opportunities for the survival of animals that lived near these groves (Panda et al., 2020).

This discriminating study revealed that honey bees are also showing habitat selection specificity and preferring the sacred trees, which are unmolested and protected by religious thoughts. Honey bees are also feeling protected in the sacred trees and all the sacred trees in the study area are saturated with more than 10-20 beehives in each where spiritual and religious rituals being performed. Moreover, previous studies suggest that traditional taboos in sacred groves help to protect various species otherwise considered as dangerous or pests like snakes in Western Ghats, India, the sclater's monkey (*Cercopithecus sclateri*) in Nigeria, rhesus macaques (*Macaca mulatta*) in India, as well as the snow leopard (*Panthera uncia*) in China (Li et al., 2014; Saraswat et al., 2015; Baker et al., 2018; Yuan et al., 2020). In this study area traditional taboos in sacred groves provide a similar type of protection to honey bee colony as well as other animals.

CONCLUSION

The present study can conclude that traditions and beliefs protect local forest patches as well as protect various animals. So, sacred groves are very significant in conserving endangered, threatened and vulnerable species of flora and fauna from being extinct. Sacred groves and sacred sites are now being challenged by many economic and social issues, and thus degradation of such type of SGs also reduces the chance of natural habitat of many animals. So SGs have immense importance for conserving animals. In this area honey bee, *Apis dorsata* populations are conserved through years in these sacred trees. So, for conserving such animals' government and non-governmental organizations should take up some strategies with the help of local people to conserve these holy places for the conservation of local flora and fauna and betterment of humankind. Similarly, more research on the specific site selection for hive formation and behaviour of honey bees are needed for understanding their behaviour and successful conservation for honey bees and other local fauna.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Optimization of Biomass Culture Yield in the Callus Culture of Mucuna pruriens Using Synergetic Combination of Auxins and Cytokinins

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ABSTRACT

The *Mucuna pruriens* (L.) DC is an important legume cover crop and it is commonly known as cow-age or cowitch or velvet bean or Alkushi, a medicinal plant traditionally used in Indian medicine which belongs to the family Fabaceae. Almost all the parts of the plant are reported to contain L-3,4-dihydroxy phenylalanine (L-DOPA). L-Dopa have high medicinal value and necessary characteristics features like high content of protein, easy digestibility similar to other pulses like soybean, rice bean, and lima bean. Therefore, it is also considered as rich source of food. Studies on its micro-propagation are up taken on high rate. In the present study, a rapid and efficient protocol for the large-scale propagation of a potential medicinal plant, *Mucuna pruriens* (L.) DC, through *in vitro* culture of different explants is described. Here we report a rapid and reliable method for high fidelity micro-propagation. The explant used under the study includes the node, internode, shoot tip and seed that were inoculated on the MS basal media after proper surface sterilization. The explants were incubated on the MS basal media supplemented with variable concentration of the growth regulators to determine the optimal concentration for each type of explant. The study concluded that the combination of auxin and cytokinin are very much efficient for the higher yield micropropagation of explants under the *in vitro* conditions. The higher concentration of these growth regulators could also stun the growth of growing callus hence optimum concentration knowledge is a must to get maximum propagating callus from any explants used under the study. Further study could focus on applying these optimum culture conditions for the propagation of explants of *Mucuna pruriens* (L.) DC and analyze their secondary metabolites which confers commendable medicinal importance.

KEY WORDS: MICROPROPAGATION, EXPLANT, *MUCUNA PRURIENS* (L.) DC., AUXIN, CYTOKININ.

INTRODUCTION

3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) is one of psychoactive drug component which has its source from many natural food products and herbaceous plants. It acts as a precursor for many neurotransmitters likes dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) collectively known as catecholamines. D. One of the examples of herbs that is a rich source of L-Dopa is *Mucuna pruriens* (L.) DC seeds. It is commonly called velvet bean belongs to the family Fabaceae and is a commercially important medicinal plant found in bushes and hedges, at damp places, ravines, and forests of Western and Eastern Ghats of India. India is one of the natural centers of origin of the *Mucuna* in the world. Rich genetic diversity coupled with wide-ranging traditional knowledge on various usage practices offers great scope for biotechnological improvement of the *Mucuna* species

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of India for diverse applications. Besides its role in the formation of a variety of neurotransmitters, it has an impressive position in the treatment of Parkinson's disease and mental disorder. This species possesses valuable medicinal properties, and in the markets across the world, there is a heavy demand for *Mucuna* seeds containing L-DOPA (Eilittä et al., 2002, Ali et al., 2005; Alam and Anis, 2019).

Its demand in international markets has increased many folds in recent years. Since it is being used as a source of L-DOPA by many pharmaceutical companies in India, a continuous supply of L-dopa producing seeds are very much in demand. These seeds are generally collected from forests, leading to the problem of quality control especially of % of L-dopa content. The other hurdle in the collection and in its cultivation is the presence of trichomes (hairs) on the pods, which give a very strong itching sensation. Therefore, attention has been diverted to *in vitro* culture of *Mucuna* similar to *Stizolobium* which is also a rich source of L-DOPA. Micropropagation is one of the innovative methods of asexual propagation that has proved to be effective for *in vitro* propagation of medicinal and aromatic plants and for commercial exploitation of valuable plant-derived pharmaceuticals. *In-vitro* cell culture techniques are one of the innovative and effective methods to produce medicinal and aromatic plants for commercial exploitation of valuable plant-derived pharmaceuticals.

Table 1. Response of Node (N) / Internode (IN), Shoot-tip (ST) and Seed explants of *Mucuna pruriens* (L.) DC. on MS basal media supplemented with effective growth hormone during the explant preparation

Growth Regulators (mgl^{-1})	Explants	Number of Explants Cultured	No. of Responded Plant	% of Response	Response
2,4-D ($3mgl^{-1}$)	Node	10	8	80	Callusing
	Internode	10	6	60	Loose callus
	Shoot-tip	10	7	70	Crystalline callus
	Seed	10	7	70	Apical Bud
NAA ($4mgl^{-1}$)	Node	10	8	80	Well-developed callus
	Internode	10	7	70	Callusing
	Shoot-tip	10	7	70	Callus
	Seed	10	6	60	Apical Bud
BAP ($2.5mgl^{-1}$)	Node	10	7	70	Callusing
	Internode	10	6	60	Hydrated callus
	Shoot-tip	10	8	80	Shoot development
	Seed	10	5	50	Budding
Kn ($2.5mgl^{-1}$)	Node	10	8	80	Multiple shoot development
	Internode	10	6	60	Callusing
	Shoot-tip	10	6	60	Clear callus
	Seed	10	6	60	---

These techniques are under spot light for research and studied thoroughly with various combinations like plant hormones, nanoparticles and many such strategies (Rout, 2002; Faisal et al., 2005, Alam and Anis, 2019). In view to above mentioned difficulties and some of the strategies employed earlier, callus culture has been an alternative and efficient source for the production of secondary metabolites. The *in vitro* technology could be a cost-effective means of high-volume production of the elite planting material throughout the year, without any seasonal constraints (Raaman et al., 2013; Oviedo-Silva et al., 2018; Alam and Anis, 2019; Rakesh and Praveen, 2020).

MATERIAL AND METHODS

For the collection of plant material, the healthy wild plants of *Mucuna pruriens* (L.) DC. were collected from the local area of Hathwa and plain area of Kuchikot of District Gopalganj. The experimental plant is found in these areas among the hedges and bushes of other plants and the plant gives flowers and fruits in the period of October-March. The plant samples were collected, cleaned thoroughly and sorted.Explant material and sterilization: In the present work four different explants were taken from the plant including the node, internode, shoot tip and seed. The seeds were collected from the young pods of 5 months old plant and rests were collected from the in vivo grown plants and all of these were used as the explant. All the segments taken as explants were surface sterilized by cleaning thoroughly under the running tap water for 30 minutes and then were washed with a solution of labolene (5-6 drops in 100ml of water) for 5 minutes followed by washing with

distilled water. These cleaned explants were treated with 70% ethanol for 2 minutes followed by treatment of 0.1% mercuric chloride treatment for 5 minutes under the aseptic conditions. Finally the explants were washed 7-8 times with sterile distilled water to remove traces of any sterilizing agent.

Table 2. Response of different explants of Mucuna pruriens (L.) DC. on MS basal media supplemented with variable conc. of 2,4-D growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
1.5	Node	10	06	60	1.83
	Internode	10	05	50	1.54
	Shoot tip	10	07	70	1.96
	Seed	10	06	60	1.89
2.5	Node	10	06	60	1.98
	Internode	10	06	60	1.75
	Shoot tip	10	07	70	1.98
	Seed	10	06	60	2.01
3.5	Node	10	06	60	2.89
	Internode	10	05	50	2.76
	Shoot tip	10	08	80	2.89
	Seed	10	06	60	2.76
4.5	Node	10	07	70	3.05
	Internode	10	05	50	2.89
	Shoot tip	10	09	90	3.89
	Seed	10	07	70	3.85
5.0	Node	10	04	40	1.56
	Internode	10	03	30	1.23
	Shoot tip	10	06	60	1.80
	Seed	10	04	40	1.69

Culture media and conditions: The analytical grade chemicals obtained from Hi-Media laboratories and hormones and vitamins from Sigma-Aldrich chemicals were used for preparing the stock solutions and subsequent media preparation. Murashige and Skoog's (1962) salt with 3% (w/v) sucrose was used as basal medium excepting seed germination medium, which completely lacked sucrose. After adding the growth regulators, the pH of the medium was adjusted to 5.7±0.1 followed by gelling with 0.8% of agar in case of solid medium. The media was autoclaved at 121°C for 20 minutes. All the cultures were incubated in a growth chamber maintained at a temperature of 25±2°C, relative humidity, 70-80% and photoperiod of 16:8 h duration under photon flux density of 50 µE mol m-2s-2 provided by day light fluorescent tubes.

Explants preparation: All the sterilized explants materials were used for the preparation of explants that can be used for callus induction. The surface sterilized materials were germinated on MS basal medium without sucrose and the seedlings were allowed to grow for 12-14 day (d) until the auxiliary buds become prominent. The explants were then prepared as per the technique described by Jayanand et al. (2003). Auxiliary buds measuring 0.8-1.0 cm lengths were aseptically inoculated onto MS medium supplemented with various shoot induction hormones.

Initiation of Callus: The auxiliary buds obtained were aseptically transferred on the MS basal media containing various concentration of 2,4-D (1.5, 2.5, 3.5, 4.5, 5.0mg/L), NAA (2.0, 3.5, 4.5, 5.5, 6.5mg/L), BAP (0.5, 1.5, 2.5, 3.5, 4.5mg/L), and Kn (1.5, 3.5, 5.5, 6.0, 7.5mg/L) for the callus induction process. Primary callus was established from cotyledonary leaf explants. For secondary callus production, a small portion of primary callus was excised by using sterile knife holder and was sub-cultured periodically once in three weeks. The secondary callus was used for all the experimental studies. A standard approach of Latin square method was followed for screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2,4-D and NAA) and cytokinins (BAP and Kn) alone and in combinations (Collin and Edwards, 1998).

Callus growth: The growth measurement of callus was determined by standard method. The growth of the callus and its biomass was measured in terms of fresh (FW g/L) and dry weight (DW g l^{-1}). FW of callus was measured after removing the excess moisture and agar adhering to the callus surface using blotting paper. DW of callus was

determined by drying the callus in hot air oven at 60°C for 24 hr and was expressed in g l^{-1} DW culture.

Acclimatization: Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing sterile garden soil supplemented with the combination of growth regulators, under diffuse light (16:8 h photoperiod) conditions. Potted plantlets were covered with a transparent polythene membrane to ensure high humidity and watered every three days with half-strength MS salt solution for two weeks. Polythene membranes were opened after two weeks in order to acclimatize plants to field conditions. After four weeks, acclimatized plants were transferred to pots containing normal soil and maintained in a greenhouse under normal day length conditions.

Table 3. Response of different explants of Mucuna pruriens (L.) DC. on MS basal media supplemented with variable conc. of NAA growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
2.0	Node	10	07	70	2.43
	Internode	10	05	50	2.10
	Shoot tip	10	08	80	2.89
	Seed	10	07	70	3.01
3.5	Node	10	08	80	2.13
	Internode	10	06	60	1.98
	Shoot tip	10	07	70	3.52
	Seed	10	06	60	3.89
4.5	Node	10	07	70	2.52
	Internode	10	07	70	2.76
	Shoot tip	10	08	80	3.95
	Seed	10	07	70	2.89
5.5	Node	10	08	80	3.85
	Internode	10	06	60	3.65
	Shoot tip	10	09	90	2.56
	Seed	10	08	80	3.85
6.5	Node	10	06	60	2.89
	Internode	10	05	50	2.65
	Shoot tip	10	06	60	2.80
	Seed	10	06	60	2.69

RESULTS AND DISCUSSION

For the present work, four different explants were selected including the nodes, internodes, shoot tips and seeds of the plant *Mucuna pruriens* (L.) DC. First of all the selected parts were sterilized and inoculated on the MS basal media in order to analyze their response and to use them for the preparation of explants for callus induction. For the following work ten replicates from each one was cultured and their response on the media was recorded. All of them were cultured on MS basal media supplemented with different concentration of different growth regulator. The response of nodes, internodes, shoots tips and seeds on the different media are summarized in the table no.1. The results obtained showed that all the selected parts have shown the response in the range of 60-70% on all the four growth regulator media. The characteristic of the resulting callus is also mentioned. These growing calli were used for the explant's preparation and callus induction on the primary culture (Oviedo-Silva et al., 2018).

All the explants now prepared from the different parts were carried forward for the process of callus induction in order to obtain the primary culture. These explants were inoculated on the MS basal media that was supplemented with variable concentration of different growth regulators separately. Different concentration of each selected growth regulator was taken in order to optimize the best concentration at which the percentage response is maximum. For each explant and each hormone five different concentrations were taken with 10 replicates. The result obtained from this optimization study is listed in the table no. 2, 3, 4, 5 for the different concentrations of 2,4-D, NAA, BAP and Kn respectively. The table summarizes the result of 10 replicates giving information about the % response and average fresh weight of the callus for each (Oviedo-Silva et al., 2018).

Table 4. Response of different explants of Mucuna pruriens (L.) DC. on MS basal media supplemented with variable conc. of BAP growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
0.5	Node	10	07	70	1.54
	Internode	10	06	60	1.23
	Shoot tip	10	08	80	2.89
	Seed	10	07	70	3.01
1.5	Node	10	08	80	2.13
	Internode	10	06	60	1.98
	Shoot tip	10	07	70	3.89
	Seed	10	06	60	4.15
2.5	Node	10	08	80	3.05
	Internode	10	07	70	2.76
	Shoot tip	10	08	80	3.68
	Seed	10	07	70	3.85
3.5	Node	10	08	80	3.85
	Internode	10	06	60	3.65
	Shoot tip	10	09	90	3.56
	Seed	10	08	80	3.85
4.5	Node	10	07	70	2.89
	Internode	10	05	50	2.65
	Shoot tip	10	06	60	2.98
	Seed	10	06	60	2.96

The overall results obtained from this analysis and the data obtained clearly depicts that there is a very variable requirement of these growth regulator for the different explants. The concentration at which the maximum response was obtained for the different explants is as, for internode explant 2.5 mg 2,4-D, 4.5 mg NAA, 2.5mg BAP and 5.5 mg Kn. per litre of the media; for node explant 4.5mg 2,4-D, 5.5mg NAA, 3.5mg BAP and 5.5 mg Kn; for seed and shoot tip explant 4.5mg 2,4-D, 5.5mg NAA, 3.5mg BAP and 6.0mg Kn.

The results obtained depicted the optimization of the growth regulator's concentration required to obtain the better growth response of *Mucuna pruriens* (L.) DC. under in vitro conditions. The average fresh weight of the callus has also shown better response at the suitable hormonal concentration. After this study the next approach was the acclimatization step that is used for the field transfer of the plantlets obtained under in vitro process. For this the soil was collected from the area from where the sample plant was obtained. The soil was supplemented with growth regulator combination and the plantlets were transferred in the pots and kept under the regulated conditions for 25-30 days. The result obtained is summarized in the table no. 6 depicting the percentage response for all explants as well as the characteristic features of the plantlets growing in the soil (Oviedo-Silva et al., 2018).

The results obtained from the study clearly depicted that the growth regulators have played an important role in maintaining and promoting the proper growth of the explant under the in vitro environments. The importance and need of these growth regulators is clearly depicted from the study as well as there is a need for implementing these hormones in order to obtain maximum yield of the work. The stimulating effect of BAP on multiple shoot formation has been reported earlier for several medicinal and aromatic plant species (Khalafalla and Hattori, 1999). The results obtained under the study have clearly established the consistency with other findings where the addition of NAA promotes the proliferation and elongation of shoots in *Petasites hybridus, Eucalyptus grandis*, and *Hybanthus enneaspermus* (Wildi et al., 1998; Cid et al., 1999; Prakash et al., 1999). Higher concentration of NAA (1.0 µM) suppressed the shoot regeneration and resulted in basal callusing of the various hormones tested, BAP alone was more effective over Kn and other combinations of hormones (Alam et al., 2020).

Superiority of BAP over Kn for multiple shoot formation was also demonstrated in Pterocarpus marsupium (Chand and Singh, 2004). The study observed that there is no directly proportional relation between the concentration of hormones and percentage response as the better response was obtained at an optimal concentration nor too low nor too high.

Table 5. Response of different explants of *Mucuna pruriens* (L.) DC. on MS basal media supplemented with variable conc. of Kinetin growth regulator with a culture period of 21 days and 10 replicates

Conc. (mgl^{-1})	Explant	No. of explants cultured	No. of respond explants cultured	%of Response	Av. Fresh wt. of callus (in gm)
1.5	Node	10	06	60	1.64
	Internode	10	06	60	1.23
	Shoot tip	10	08	80	2.89
	Seed	10	07	70	3.01
3.5	Node	10	07	70	3.13
	Internode	10	06	60	2.98
	Shoot tip	10	07	70	3.99
	Seed	10	06	60	4.6
5.5	Node	10	08	80	3.95
	Internode	10	07	70	3.76
	Shoot tip	10	08	80	3.68
	Seed	10	07	70	3.85
6.0	Node	10	07	70	2.85
	Internode	10	06	60	2.65
	Shoot tip	10	09	90	3.56
	Seed	10	08	80	3.85
7.5	Node	10	06	60	1.89
	Internode	10	05	50	1.65
	Shoot tip	10	06	60	2.98
	Seed	10	06	60	2.96

Table 6. Response of Mucuna pruriens (L.) DC. on field transfer in various concentrations of phytohormones (Auxin and Cytokinin) documented after 25-30 days of plantation in the soil

Growth hormone (mgl^{-1})	Explants	No .of Explants Transferred	No. of respond explants	% of Response	Responses			
					Shoot	Bud	Root	Other Remarks
	Node	10	07	70	Elongated	Compact and green	2-3 root hair	Well developed with dark green leaves
	Internode	10	05	50	Dwarf	Flattened	Short Adv. root hairs	Not showed well responsive growth
NAA and BAP	Shoot-tip	10	08	80	Straight long	Green	Multiple root hairs, white	Well developed green leaves with long stem
	Seed	10	07	70 elongation	Apical Bud	Well dev.	Elongate white multiple root	Long straight stem

	Node	10	08	80	Elongated	Compact and green	2-3 Hair root	Well developed with dark green leaves
	Internode	10	06	70	Short	Flattened	Short Adv. root	Not showed well responsive growth
NAA and Kn (4.5+5.5)	Shoot tip	10	07	70	Straight long	Green	Multiple roots hair, White	Well dev. green eaves with long stem
	Seed	10	06	60	Apical elongation	Well dev. bud	Elongate and white multiple root	Long straight stem

Cytokinin concentrations beyond the optimum range adversely affected the shoot development, as the regenerated shoots became stunted and dense. The stunted nature of shoot formation corresponding to increased concentration of BAP in the medium was also reported in Orthosiphon (Leng and Lai-Keng, 2004) and *Eupatorium* (Martin, 2003). Faisal et al. (2005) worked on three different cytokinins, 6-benzyladenine (BA), kinetin (Kin) and 2-isopentenyl adenine (2-iP) by supplementing them to (MS) medium and showed that BA at an optimal concentration of 5.0µM was effective in inducing multiple shoots. Method of *in vitro* culture has also been well studied for other L-DOPA producing medicinal plant species (Oviedo-Silva et al., 2018).

Alam and Anis (2019) have worked to conclude indole-3-butyric acid (IBA) as most suitable supplement for *in vitro* culture of this plant. The combinations of hormones that showed optimum growth in our study similar effect was seen in Alam et al. (2020) where various strength of 6-benzyladenine), Kinetin and 2-isopentenyl adenine were observed to show optimum culture conditions for *in vitro* regeneration. Thus these combinations could be used in high rate growth of *M.puriens* and used for L-DOPA production (Alam et al., 2020).

CONCLUSION

In conclusion, a cell culture methodology for better propagation under *in vitro* condition is found to be highly useful for commercial production of medicinally important compounds. The present study developed an efficient and optimum callus biomass using a synergetic combination of auxins and cytokinins. The study concluded the optimal normal concentration for better and enhanced production of the plantlets from tissue culture technique. The study shows a better the large scale application of such finding for the propagation of medicinally important plant on large scale in order to obtain their metabolites or compounds for further study and uses. Further studies will be directed toward large scale production, testing the efficacy of secondary metabolites through animal cell lines and exploring market potential.

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Distribution and Hydrolytic Potential of Bacteria During Monsoon and Post Monsoon Seasons in the Mangrove Sediments of North Kerala

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ABSTRACT

Mangroves are coastal ecosystems, found in tropical and subtropical regions around the world. Life in mangroves requires special adaptations to survive due to high moisture, high salinity, and hypoxia conditions which breed many kinds of novel organisms. Such complex ecosystems harbour diverse groups of microorganisms including bacteria, actinomycetes, fungi etc. The present study focuses on the distribution and hydrolytic enzyme potential of culturable bacteria from mangroves of Northern Kerala with respect to changes in seasonal and physico chemical characteristics. Sediment samples were collected from 8 locations in 5 districts along the northern coast of Kerala, during monsoon and post monsoon seasons of 2018-19. pH of the sediments varied between 6.4 – 7, temperature between 20-29°C and organic matter between 0.2%± SD to 4.1%± SD. The colony forming unit per gram (CFU/g) of bacterial isolates showed a radical variation between the seasons. 66% of the isolates showed various hydrolytic enzyme activities during monsoon while 87% in post monsoon. The current study demonstrates the seasonal variation in microbial count and their enzyme activity related to a seasonal shift in availability of substrates. Monsoon season shows increase in number and were as post monsoon shows the hydrolytic potential of bacteria. Mangroves provide a rich resource for the discovery of potential bacterial species capable to produce various extracellular enzymes that could be used for human life, agriculture, industry and bioremediation etc. The study shows the influence of rainfall in distribution and bioactivity of bacteria in the unique mangrove ecosystem.

KEY WORDS: MANGROVE, BACTERIA, POPULATION, HYDROLYTIC POTENTIAL, ORGANIC MATTER.

INTRODUCTION

Mangroves are amongst the most productive marine ecosystems on earth, providing a habitat for many species and key goods and services for human beings. Mangroves, sea grass meadows and salt marshes collectively termed "Blue forests" are counted among the most vulnerable and productive coastal ecosystems on the planet. Mangrove forests evolved where two most contrasting environments meet – the interface between the land and sea. Mangroves acts as a natural barrier against sea level rise and coastal flooding, apart from providing numerous other ecosystem services such as carbon sequestration, climate regulation (Brander et al., 2012; Barbier, 2016; Himes et al., 2018). Kerala was once blessed with these amazing ecosystems covering about 700 km^2 till 1957, but it is now in a drastically declined state reducing to less than 17 km^2. Despite the breadth and quantity of services that mangrove ecosystems provide, that are being degraded at an alarming rate, the continuous threat is from the consequences of rapid urbanization and population change. India contains nearly 3.3 percent

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of this mangrove habitat (MEA, 2005; Tallis et al., 2012; Ghosh, 2019).

Sediment microorganisms play important roles in the mangrove ecosystem and make essential contributions to its productivity. Bacteria create mutualistic relationships with mangrove flora. The bacteria provide services such as N-fixation while the mangroves trees provide root exudates, stimulating microbial growth activity. There is also competition among the microorganisms because of the limited amount of nutrients available in mangroves. All of these things together make mangrove microbes highly efficient nutrient cyclers. Bacteria plays important role in nitrogen fixation, sulphate reduction, phosphate solubilization and methanogenesis (Liang et al., 2007; Ghosh, 2019).

Decomposition of mangrove vegetation is carried out by organisms such as crabs, fungi, bacteria, protozoa, and microalgae. Crabs relocate and macerate the fallen leaves while the other microorganisms decompose the leftover material through the use of enzymes such as cellulase, pectinase, protease, and amylase. Most of soil organic matter is derived from the vegetation that is partly disposed on the soil surface as an organic layer (litter) and partly distributed into the soil (Klein, 2000; Regina and Tarazona, 2001; Liang et al., 2007). The main factors that control the organic matter transformation process are: the quantity and quality of litter material components, the physical and chemical environment, and the decomposition organisms (Swift et al., 1979). Among the soil organisms, the bacteria and fungi present the highest values of biomass and respiratory metabolism, and have greater participation in the organic matter decomposition process (Persson et al., 1980; Liang et al., 2007).

Bacteria represent the major group, responsible for 25 to 30% of the total soil microbial biomass. Increase in the bacterial community during the summer have been attributed to increase in air temperature (Chhonkar and Tarafdar, 1984); however, decreases fungi and increases on bacteria relative quantities, respectively, were observed with increasing soil fertility. The soil microbial enzyme activity is affected by edaphic and climatic factors (Jha et al., 1992; Pennanen et al., 1999). Microbes constitute the largest pool of metabolic pathways on earth with potential biotechnological and environmental implications. In 1988, Alongi reported that in tropical mangroves, 91% of the total microbial biomass is bacteria and fungi, another 7% is algae, and 2% is protozoa. Microbial diversity of mangrove ecosystems provides information on their ecological role and unique biotechnological potential in the field of agriculture, industry, medicine, and pharmaceuticals (Lageiro et al., 2007).

In India, particularly in Kerala, there is lack of studies about the mangrove microbes, despite its vital role in this peculiar ecosystem. The present study attempts to investigate the distribution and hydrolytic enzyme potential of culturable bacterial isolates from the mangroves of Northern Kerala during the monsoon and post monsoon seasons of 2018-19. Currently Kannur and Kasaragod districts in Kerala are having the maximum coverage of mangroves (Mohandas et al., 2014).

MATERIAL AND METHODS

Sediment samples collected from the mangroves of the 5 districts along North Kerala coast from 8 sites (Fig. 1), Chandragiri (KGD) - 12°05'32" N 75°13'39" E, Edat (EDT) - 12°05'32" N 75°13'39" E, Pazhayangadi (PYD)- 12°02'72" N 75°29'31" E, Valapattanam (VPT) - 11°93'45" N 75°35'35" E, Elathur (ELR) - 11°19'43" N 75°45'2" E, Kadalundi (KDI) - 11°07'43" N 75°49'48" E, Ponnani (PON) - 10°47'1" N 75°55'3" E and Chettuva (CTV) - 11°1'41" N 75°52'6" E.

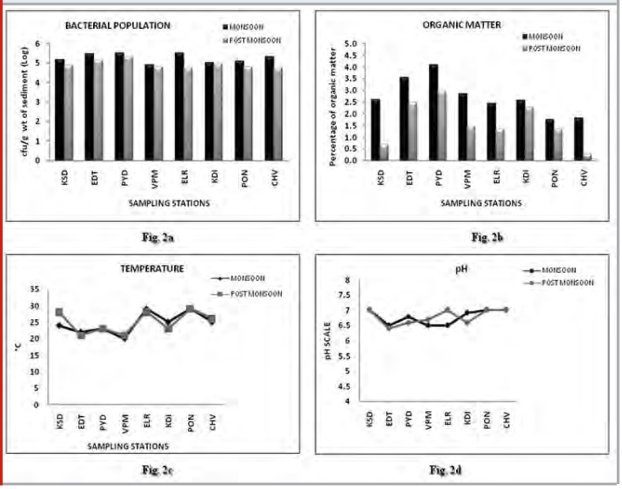
Figure 1: Map of Kerala showing the sampling sites of mangrove sediment

Scale bar 1mm = 1 Km

Samples were collected from same spot during two periods of the year viz., monsoon (June – September, 2018) and post monsoon (November – February, 2018-19). Sub surface (0-15 cm) samples (approximately 10-20 gm) were collected using hand core method and transferred aseptically into sterile polythene bags, transported in ice boxes and processed within 4 hrs of

collection. A total of five sub-samples collected from each location and pooled for the analysis. A fraction of the sediment sample was stored at -20°C for biochemical analysis. Temperature and pH of the sediment were measured during the time of collection using portable digital pH meter and mercury thermometer respectively. Organic matter was analyzed in triplicates using modified Walkley and Black method. The collected soil samples were serial diluted (10^{-1} to 10^{-3}) and plated to nutrient agar medium employing spread plate method. The plates incubated at 28 ± 2° C for 24 hours. The developed colonies were purified and transferred to nutrient agar slants for further analysis (Trivedi, 1986).

Figure 2: Variations in the population of cultivable bacterial colonies (CFU/g) and physico-chemical parameters of sediment (Total organic matter, Temperature and pH) from different mangrove sites during monsoon and post monsoon seasons



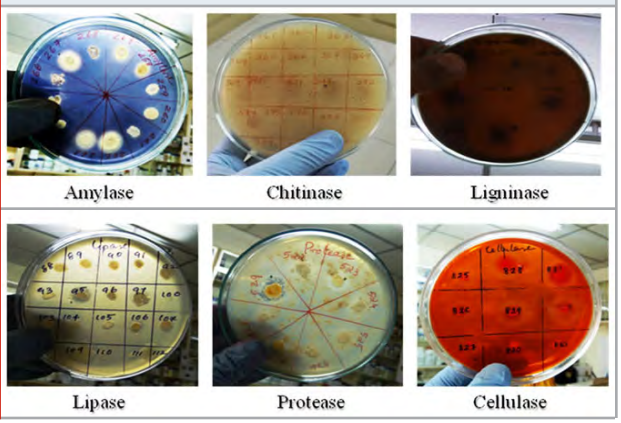
The bacterial isolates were screened for their capacity for the production of hydrolytic enzymes viz. protease, amylase, lipase, cellulase, ligninase, DNase and laccase as per the standard methods. Nutrient agar supplemented with casein (2%), starch (1%) and tributyrin (1%) were used for detection of protease, amylase and lipase respectively. DNase agar, Cellulase agar, agar supplemented with 0.01% guiacol and Crawford's agar supplemented with 0.5% tannic acid were used for the detection of DNase, cellulose, laccase and ligninase activity respectively. The plates were spot inoculated and incubated at 28 ± 2°C overnight. Formation of clearance/ halo zone or brown colour around the colonies indicates positive result.

RESULTS AND DISCUSSION

A total of 253 bacterial isolates were obtained, studied and stored during the collection period in which 153 isolates were obtained during monsoon and 100 isolates during post monsoon. The colony forming units per gram (CFU/g) of the diluted sediment sample from each of the duplicate plates were calculated and the average number was taken into consideration. The CFU/g of bacteria in mangrove sediments showed significant difference between the sampling periods (Fig. 2a). During monsoon, maximum CFU/g was found in the mangrove sediments from Pazhayangadi (310000) while those from Valapattanam showed the minimum CFU/g (78000). The trend was slightly different during post monsoon period where the sediments from Pazhayangadi (208000) showed high while those of Chettuva showed low bacterial count (54000). In all mangrove stations CFU/g decreased from monsoon to post monsoon (Vidya and Sebastian, 2020).

The physico chemical parameters of the sediments like pH and temperature of the mangrove sediments were recorded from sampling sites during the time of collection. The organic matter content was determined later in the laboratory. The total organic matter content in the sediment varied between 0.2% ± SD to 4.1% ± SD (Fig. 2b), temperature between 20-29 °C (Fig. 2c) and pH between 6.4 -7 (Fig. 2d), during the period of study. Organic matter content was high in Pazhayangadi both in monsoon (4.1% ± SD) and post monsoon (3% ± SD) and low in Ponnani (1.7% ± SD) during monsoon. But, in post monsoon, Chettuva (0.2% ± SD) showed lower values. Generally, the organic matter was found to higher in the monsoon than in post monsoon (Vidya and Sebastian, 2020).

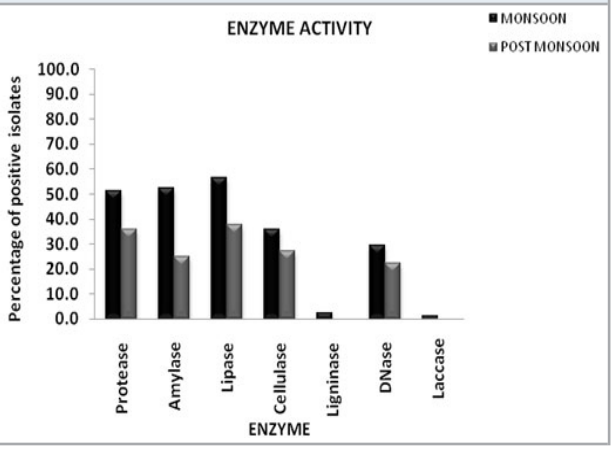
Figure 3: Culturable bacterial isolates from mangrove sediments showing various enzyme activities



The hydrolytic enzyme potential of bacterial isolates during the study period showed considerable variations between the monsoon and post monsoon season (Fig. 3). The number of isolates showing enzymatic potential in each season is calculated and expressed as percentage. The results showed that the number of bacterial isolates having hydrolytic activity increased during post monsoon compared to monsoon season. 66% of the isolates showed various hydrolytic enzyme activities during monsoon and 87% in post monsoon. Significant changes were found in the pattern of enzyme activity

showed by the bacterial isolates between two seasons. The order of the enzyme activity showed by the bacterial isolates during monsoon was found to be lipase> amylase>protease>cellulase>DNase>ligninase>laccase and that of post monsoon lipase>protease> cellulase>amylase> DNase (Fig. 4).

Figure 4: Percentage of bacterial isolates showing various enzyme activities during monsoon and post monsoon



Lipase and protease enzyme activity were shown by maximum number of isolates while ligninase and laccase activity by minimum number of isolates during the study period. There was no ligninase and laccase producing isolates obtained during post monsoon while 2.6% isolates showed ligninase and 1.3% isolates showed laccase activity (Vidya and Sebastian, 2020).

The present study showed increase in the CFU/g of cultivable bacteria from the sediments of all the 8 mangrove stations during monsoon when compared to post monsoon period. The percentage of organic matter in the mangrove sediments from all the sites studied showed increasing pattern from monsoon to post monsoon. The increase in the moisture content and organic matter might be the reason for enhancement of bacterial count in monsoon season. Several studies showed that microbial population varied based on moisture and temperature changes among seasons and are the main factors related to microbial abundance and distribution (Kutty and Philip, 2008; Luo et al., 2019; Vidya and Sebastian, 2020). The reported relationship between soil physicochemical properties and microbial communities varies among studies. In some ecosystems, soil microbes grow rapidly via energy and nutrition consumption (Huang et al., 2005; Huang et al., 2013).

Organic matter content is high in Pazhayangadi both in monsoon and post monsoon, it may be due to the climate and sediment changes of mangrove station. In fact, the factors that trigger rapid propagation of microbes most likely are high moisture, warm temperature, appropriate pH and outside carbon input. Some of the microbes consume carbon and nitrogen sources from outside, some likely get nutrition in soil, and some of them get energy from metabolites of other microbial groups. Most importantly, several key microbial organisms living in the habitat even effect the whole interaction between microbial community dynamics and soil chemical properties. It is hardly conclusive because the function of many key sediment microbes remains unclear. In this regard, extensive studies are needed for understanding the interrelationship between microbial distribution and diversity along with all components of the microbial community and the physico chemical properties (Luo et al., 2019).

The number of bacterial isolates showing various enzymatic activities was higher during post monsoon season. Previous studies have demonstrated a seasonal variation in microbial enzyme activity related to a seasonal shift in availability of substrates and a seasonal variation in soil temperature and moisture (Kaiser et al., 2010). Seasonal and experimentally induced alterations in resource availability and abiotic factors, however, have also been shown to induce changes in microbial community composition and hydrolytic enzyme potential (Lauber et al., 2008; Kaiser et al., 2010; Fierer et al., 2012). In the present study also, considerable variations were observed in microbial populations and enzyme activity during the two seasons and also between the sampling stations, which substantiate with the studies of Vidya and Sebastian (2020).

Majority of the bacterial isolates from the mangrove sediments studied were lipolytic and proteolytic. This shows the presence of lipid and protein substances and its metabolic processes in the sediments. The extracellular enzymes produced by bacteria have variety of applications in industrial processes. Many studies reported that microorganisms possess distinct enzyme production ability to degrade complex hydrocarbon mixers such as crude oil from industrial waste water, fresh water and marine environments (Silva et al., 2015; Safitri et al., 2015). Bacteria are the most dynamic agents in petroleum degradation and they perform as primary degraders of spilled oil in various environments (Wang and Shao, 2012). Microbial enzymes have applications in the detergent, food, flavor, pharmaceutical, agrochemical, chemical and cosmetic industries. Enzymes are also use effectively in bioremediation of environments contaminated with hazardous substances (Dash et al., 2013; Willsey and Wargo, 2015; Raveendran et al., 2018). Since a good number of mangrove bacterial isolates during our study showed considerable amount of enzyme activities, they can be further optimized for the large-scale production (Raveendran et al., 2018).

Due to unique environment, mangrove inhabitants develop adaptations for their growth and survival.

Mangrove bacteria also show unique enzymatic properties (Gomes et al., 2011). This needs research attention and must be explored for the discovery of novel bioactive compounds. The current study focuses on the bacterial richness in the mangrove habitat and their hydrolytic enzyme potential. The study attempts to highlight the importance of mangrove ecosystem and the preservation. The findings from our study suggest that monsoon season harbored more bacterial species in mangrove sediments compared to other seasons. This is due to the environmental and physico chemical characteristic changes in the habitat. Further, the promising bacterial isolates showing higher enzymatic potential will be identified and optimized for future applications (Gomes et al., 2011; Raveendran et al., 2018).

Sampling stations: KSD - Kasaragod, EDT - Edat, PYD - Pazhayangadi, VPM - Valapattanam, ELR - Elathur, KDI-Kadalundi, PON – Ponnani, CHV-Chettuva

CONCLUSION

The present study focuses on bacterial isolates from the mangrove sediments of Northern Kerala, their enzymatic potential and sediment ecology during two seasons of the year viz monsoon and post monsoon. The number of cultivable bacteria and their hydrolytic enzyme production were found to be significantly affected by the moisture content and the organic matter present in the sediment. As the mangroves are unique ecosystem with extreme characteristics, more attention has to be given so as to explore novel bioactive compounds which can be applied in industries and also in the management of mangrove ecosystem. The present study thus provides light to the need for extensive studies in mangrove habitats for the conservation and management of this unique ecosystem.

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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Characterization of Different Cell Types in the Pituitary Gland of Indian Fresh Water Spiny Eel *Mastacembelus armatus* (Lacepede)

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ABSTRACT

Mastacembelus armatus is an indigenous fish species of southern Asia that also resides in the Indian subcontinent. This fish species is facing an alarming decline in their number in the last decade. Due to its moderate cost, it is mainly taken by the lower income group of people within the society. The reproductive care, by artificial breeding, has been taken for those fish species having a high cost in the market or becoming less in number in nature for business purposes or preserving the biodiversity, respectively. The present study was undertaken to characterize different cell types in the pituitary gland because these are ultimately responsible for the maintenance of pituitary-gonadal endocrine cascade. This work has been done purely on histological techniques. In the present investigation the adenohypophysis is divisible into three component parts viz. antero – dorsal rostral pars distalis (RPD), the middle proximal pars distalis (PPD) and the posterior massive pars intermedia (PI). The acidophilic prolactin cells and ACTH cells are found in the RPD, basophilic GTH cells, TSH cells and acidophilic STH cells are found in PPD whereas MSH and MSH cells are found in PI regions. The neurohypophysis in *M. armatus* is composed of axonal fibers originating from neuronal cell bodies in the hypothalamus. Understanding the pituitary architecture and cell types for this fish species is of immense importance to save this indigenous variety by artificial breeding, which we are trying to discuss in the detail within this paper of ours.

KEY WORDS: ACIDOPHILIC, ADENOHYPOPHYSIS, MASTACEMBELUS ARMATUS, NEUROHYPOPHYSIS.

INTRODUCTION

The function of pituitary is mostly controlled by the hypothalamus through the synthesis and release of gonadotropin-releasing hormone (GnRH), therefore, acting as a major initiator of the hormonal cascade controlling the reproductive axis. Gonadal activities in teleost fishes primarily depend on the function of pituitary gonadotrophs and that the pituitary and the gonads exist in a mutual state of excitation and inhibition (Farbridge et al.1985; Kaneko et al., 1986). The hypothalamo-hypophyseal complex in vertebrates with their neurosecretory nuclei and long axons, is a coordination point in the vertebrate brain and is known to involve in a complex interaction of a variety of neurotransmitters which modulate the influence of several trophic hormones by controlling their active secretion by releasing or inhibiting hormones within the hypophysis itself (Peter et al., 1991).

Pituitary gonadotrophic hormones and GnRH are important in implicating these hormones in gonadal

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maturation and sex steroid production which plays a very important role in gametogenesis, final maturation of oocytes and spermiation (Parhar et al., 2003; Lethimonier et al., 2004; Chakrabarti and Chowdhury, 2015, Trudeau and Somoza 2020).

The teleost hypophysis is generally composed of a neurohypophysis and the adenohypophysis. A significant feature of the teleost pituitary is the interdigitation between the neurohypophysis and adenohypophysis, and at least in some species a prominent innervation of the adenohypophysis (Da Lage,1955). Among all groups of vertebrates perhaps the teleosts show greater structural diversity in the organization of their pituitary gland. In elucidating pituitary function a logical first step is to identify the specific type of hormone-secreting cells. The identification and distribution of the cell types in the pituitary gland of different teleosts have attracted some investigators from the histochemical, ultrastructural and immunocytochemical techniques (Ball and Baker, 1969; Holmes and Ball, 1974; Joy and Sathyanesan, 1980; Chakrabarti and Chowdhury, 2015 Hassan El-Sayyad et al. 2020).

Most of the authors have pointed out that the secretory cells of the pituitary gland show different patterns of distribution in the rostral pars distalis, proximal pars distalis and pars intermedia zones of adenohypophysis. They pointed that the identification and distribution of the different cell types indicate that the adenohypophysis consisted mainly of two groups: basophilic or PAS positive and acidophilic or PAS negative (Chakrabarti and Chowdhury, 2015). The present studies were undertaken with a view to determine the cytology of the pituitary and to identify and localize the different cell types in pituitary of freshwater spiny eel *Mastacembelus armatus* (Lacepede) by using various modern staining techniques.

MATERIAL AND METHODS

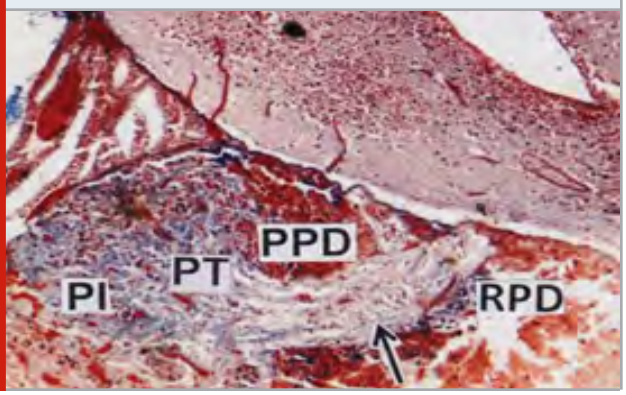
Adult male (average length 15.2 to 15.8 cm) and mean body weight (50g to 75g) and female (average length 17.5 to 17.7 cm) and mean body weight (55g to 70g) of M. armatus were procured fortnightly throughout the consecutive years from particular pond of Asansol in order to avoid ecological variations than can affect development of hypothalamus, pituitary and gonads. The fishes were collected during the second week of every month from January 2019 to December 2019. As the pituitary gland of *M. armatus* lodged inside *sella turcica*, it was difficult to dissect out the pituitary intact along with the brain. The entire brain was exposed by dissection from the dorsal aspect and subsequently immersed in 10% neutral formalin for hardening at the fish collection site.

After 45 minutes, the brain including the hypothalamus and the pituitary gland were carefully dissected out from the cranium and subsequently fixed in Bouin's fixative, Zenker's fluid and Eltman fixatives. After proper fixation, pituitary gland throughout the year were placed in 70% ethanol for overnight and subsequently dehydrated through ascending ethanol series followed by acetone and then cleared in benzene. Tissues were then embedded in paraffin wax (56^0C-58^0C melting point). Mid sagittal section and frontal section of pituitary gland along with hypothalamus were cut at 4 µm thickness using a Leica RM 2125 RT microtome. Deparaffinized sections of pituitary and hypothalamus were stained by techniques which are as follows:a) Chrome alum haematoxylin phloxin (CAHP) (Gomori 1941).b) Mallory's triple stain (MT) (Mallory,1936)c) Aldehyde fuchsin (AF) (Gabe, 1953).d) Alcian blue-orange G-acid fuchsin (AB-OFG) (Slidders,1961).Slides were examined under microscope, followed by microphotography.

RESULTS AND DISCUSSION

Nerve fibers from hypothalamus pass to the pituitary and thus connecting neurohypophysis (arrow) with the brain (Fig.1). Based on histological features and of its cell types, the adenohypophysis is divisible into three component parts viz., antero – dorsal rostral pars distalis (RPD), the middle proximal pars distalis (PPD) and the posterior massive pars intermedia (PI). Although there is no sharp demarcation between these zones but narrow line of penetration of axonal fibres of neurohypophysis ramified between RPD, PPD and PI (Fig.1). The hypothalamus consists of two nuclei rich areas viz., elongated nucleus preopticus (NPO) and oval shaped nucleus lateralis tuberis (NLT) (Fig. 1). Based on different staining techniques various cell types have been identified in the RPD, PPD and PI.

Figure 1: Pituitary gland (PT) attached to the brain. The PT is divided into rostral pars distalis (RPD), proximal pars distalis (PPD) and pars intermedia (PI), (CAHP) x 50.



The Rostral pars distalis (RPD) zone is packed closely with mostly acidophilic cells interspersed with a few basophils (Fig.2). For distinguishing the various cell types according to their morphology, size and shape of the cells and specially the stainability of their secretory granules is very important. In the RPD the acidophilic prolactin cells (PRO) occupy the major part of the RPD and stained red with acid fuchsin having densely stained rim of cytoplasm (Fig. 3). Other types of acidophilic cells which are numerically fewer and comparatively less

chromophilic are ACTH cells which are dispersed among the PRO cells (Fig. 3).

Figure 2: PT showing small RPD, moderate PPD provided with acidophils and basophils and massive PI. (MT) X 100.

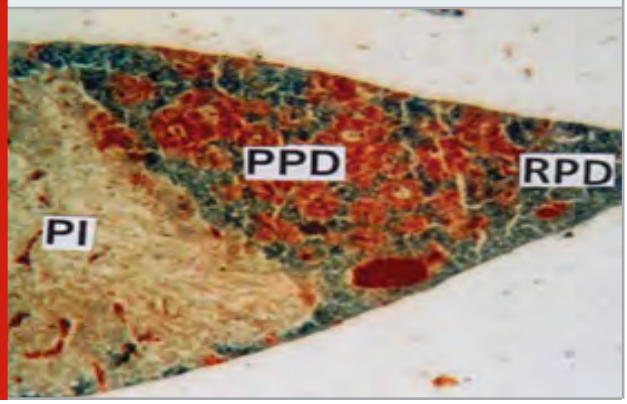
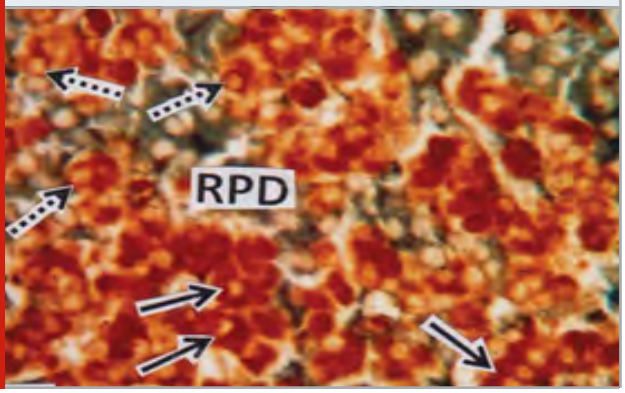


Figure 3: RPD showing tubular arrangement of acid fuchsin stained prolactin cells (PRO) (solid arrows) and dispersed ACTH cells (broken arrows). (MT) x 40



The Proximal pars distalis (PPD) area of pituitary gland is densely occupied by basophilic chromophils and showing clear innervation of axonal fibres (Fig. 4). The anterior and middle part of PPD is provided with alcian blue positive basophilic rounded or oval gonadotroph (GTH) cells. The GTH cells are intermingled with basophilic thyrotrophs (TSH) which are elongated in shape (Fig. 5) and densely stained with alcian blue. The acidophils present in proximal pars distalis are generally identified as somatotrophs (STH) which are stained with orange G. The PPD region is densely distributed by orange G positive blood vessels (Fig. 5).

In Pars intermedia (PI) area the branches of the neurohypophysis interdigitate with the PI than it does in the RPD and PPD. A considerable number of acids fuchsin positive neurosecretory materials and blood vessels of various sizes have been observed in PI region (Fig. 6). The PI contains two types of cells, the larger cells stained with aniline blue and are identified as melanotrophs (MSH) cells. The comparatively smaller cells are provided with scanty cytoplasm and stained with acid fuchsin and are identified as melanocyte concentrating cells (MCH). The pituitary in teleosts is the intricacy and extent of the contact between the nervous system and glandular components, and the division of the latter into three distinct regions, all of which may have contact with the former. The hormones produced by the pituitary gland of teleosts regulate directly/indirectly some fundamental physiological processes like growth, development and reproduction (Agulleiro, 2006).

Figure 4: PPD showing densely packed aldehyde fuchsin positive basophilic cells. Innervation of neurohypophyseal axons (arrows) in the PPD. (AF) x 150.

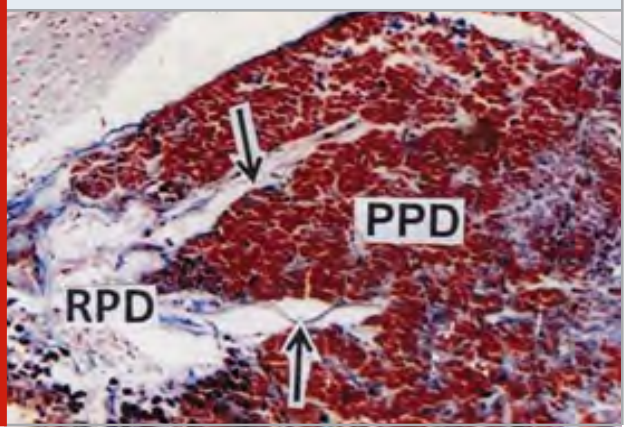
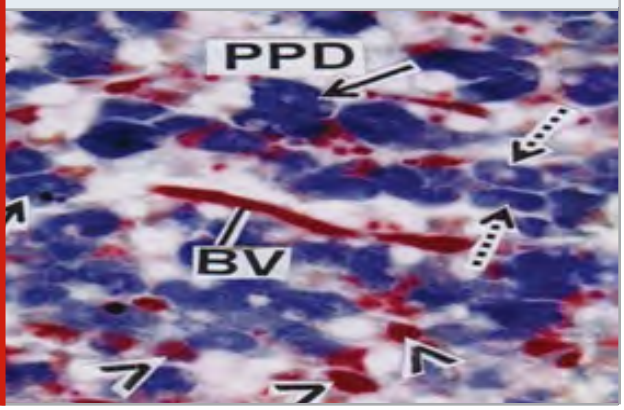


Figure 5: PPD showing alcian blue positive round or oval GTH cells (solid arrows), elongated TSH cells (broken arrows) and scattered STH cells (arrow heads). BV indicates blood vessels. (AB-OFG) x 400.

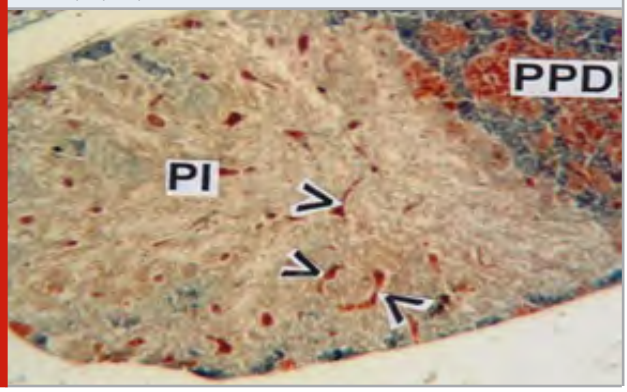


The teleostean pituitary gland is known for its remarkable structural diversity in topography, size, shape, mode of attachment in nervous component and basic histology (Green and Maxwell, 1959). The pituitary gland in *M. armatus* is small and lodged in sella turcica and situated between saccus vasculosus and brain. Based on the classical staining methods and distribution of cells the adenohypophysis consists of antero – dorsal rostral pars distalis, middle proximal pars distalis and massive pars intermedia.

In *M. armatus* the neurohypophysis is rich in blood vessels and neurosecretory materials that lie in close

proximity to the rostral pars distalis (RPD) and proximal pars distalis (PPD). Joy and Sathyanesan (1980), Jafri and Ensor (1980) identified precisely the different cell types located in the pituitary of a few teleosts. They categorized various cell types in the teleostean pituitaries on the basis of the staining reaction in the cytoplasmic content adopting different staining technologies. In *M. armatus* the RPD occupies the antero – dorsal in position of the gland and contains two types of chromophilic acidophils which show a strong affinity to acid fuchsin. The acidophilic prolactin (PRO) cells occupy the major part of RPD and the granules are stained red with acid fuchsin and are frequently attached with the blood vessels advocates their higher secretory activity. In *M. armatus* prolactin is responsible for the control of a carrier for sodium ion transport in the chloride cells of the gills, stimulation of mucus secretion both the gills and skin and is essential for osmoregulation (Aseem, 2004).

Figure 6: PI showing ramification of neurohypophyseal tract interspersed with neurosecretory materials (arrow heads). (MT) x 100.



The prolactin cells in the pituitary of *Dicentrarchus labrax* showed strong affinity to Azan stain to give red colour (Aseem, 2004). Jose and Sathyanesan (1977) and Mandal and Sinha (1985) also advocated about the random distribution of the prolactin cells in the RPD of *Labeo rohita* and *Catla catla*. The ACTH cells in the RPD tinctorially may be chromophobic or acidophilic. In *M. armatus* the carminophilic corticotrophic cells are rounded or oval in shape and generally dispersed among the prolactin cells. Mandal and Sinha (1985) reported that ACTH cells were lead haematoxylin positive and were located in the RPD bordering the neurohypophysis and occurred in groups in *Catla catla*. Zaki et al. (1996) also reported that the corticotrophic cells are generally found at the interphase between prolactin cells and neurohypophysis.The proximal pars distalis (PPD) is perhaps the most vital part of the pituitary as it shows remarkable variations in its size as well as cellular components at different reproductive phases.

In *M. armatus* three types of chromophilic cells can distinguished in the PPD on the basis of shape and tinctorial properties. However, the basophilic gonadotrophs formed the main bulk of PPD during maturation and spawning phases. Tinctorially the TSH cells in *M. armatus* closely resemble the GTH cells. TSH cells are comparatively less in number than that of GTH cells. They are also PAS positive and also stained with aldehyde fuchsin, aniline blue and alcian blue. Aseem (2004) emphasized that the TSH cells were intermingled between GTH cells in *Dicentrarchus labrax*. In *M. armatus* the main bulk of TSH cells present in the proximal pars distalis on the ventral aspect of GTH cells. Pickford and Atz (1957) noticed that thyroid follicles of hypophysectomized fish showed histological signs of inactivity. In the present observation the only acidophils stained with orange G in the PPD region considered as somatotroph (STH cells) stained acid fuchsin in some areas. These cells are dispersed among the GTH and TSH cells. This disperse nature of STH cells amongst GTH and TSH cells in *Ctenopharyngodon idella* is also reported by Hassan El-Sayyad et al. (2020).

Srivastava et al. (1977) emphasized that the acidophils of PPD are deeply stained with azocarmine and correspond to somatotrophs. The present study advocates that the pars intermedia (PI) contains two types of cells, the larger cells provided with rim of cytoplasm around nucleus which stained with aniline blue or orange G i.e. amphiphilic in staining nature and are identified as melanotrophs (MSH) cells. The comparatively smaller cells are provided with scanty cytoplasm and stained with orange G or acid fuchsin and identified as melanocyte concentrating hormone secretory cells or MCH. These cells are acidophilic in nature. The cells of pars intermedia are difficult to demonstrate uniformly in all teleosts by usual standard staining techniques. The PAS – positive MSH cells also been described in the pars intermedia of *Clarias batrachus* (Joy and Sathyanesan, 1979) and *Siganus rivulatus* (Zaki et al., 1996). Black melanophores have also been reported in zebra fish by Patterson and Parichy (2019).

The neurohypophysis in *M. armatus* is composed of axonal fibres originating from neuronal cell bodies in the hypothalamus. These nerve fibres extend as narrow strips into the pituitary gland and found to be closely associated with the blood vessels. The anterior part of the neurohypophysis penetrates the pars distalis with finger like processes, although in a less intricate manner than in the *pars intermedia*. The neurohypophysis and the neurosecretory cells of the hypothalamus form a functional unit concerned in the synthesis, transport and release of neurosecretory materials. Present findings overlap with the work of Trudeau and Somoza (2020), related to the innervations of hypothalamo-tract into the adenohypophysis.

Secretion of hormones by the different cells qualitatively and quantitatively depends on the nature of formation and discharge of specific secretory granules. The acidophil granules have a strong affinity for acid dyes of all kinds. The granules of basophil cells on the other hand contain glycoprotein as indicated by their staining properties. Parallelism between intensity of staining and hormone content has been employed to support the claim

that it is the hormone product itself of these cells which is stained. Staining reactions demonstrate the number of granules present. It is therefore, quite likely that the staining reactions are primarily due to the hormonally active product itself.

CONCLUSION

Fish production in captivity is an essential prerequisite for the increasing population globally. Indigenous fish species of the pond and ditches are the main source of protein in the rural areas of India. With demanding globalization these fish species are facing critical problem of survivalism. Induced breeding has been taken for those fish species which are economically important or number has been reduced drastically. From this research finding it has been established that in Mastacembelus armatus, different cell types are located in particular places of pituitary. The most important one is the GTH secreting cells which is present in the proximal pars distalis region. Seasonal secretion study of this particular hormone can focus light on the Induced breeding program for this fish species.

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Analyzing the Impact of Economic Shock Due to Covid19 on Consumer Behavior Pattern: A Cross Sectional Study Conducted in Delhi & National Capital Region

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ABSTRACT

As historically evident, a healthcare crisis like COVID-19 can have widespread repercussions. Under preparedness has rendered the global economy unfunctional, with businesses shutting down, massive job losses and supply chains disrupted. The current study is conceived in the backdrop of Covid19 and its impact on consumer behavior pattern particularly in the context of social, buying, traveling and spending behavior. The data was collected by circulating an online questionnaire among a selected sample based on convenient and snowball sampling technique. The data largely analyzed using quantitative techniques. Results were explained through pie-charts, bar graphs and Chi-square test in Microsoft Excel and SPSS-Version 24, respectively. The study finding shows that respondents are resorted to limited spending only. Sectors like aviation, hospitality, luxury goods and services were not given priority. These sectors have faced the brunt of reduced incomes and exhausted savings. Financial insecurity has rendered the market tight-fisted and apprehensive about new brands, whereas increased demand of domestically-made products, FMCGs and focus on hygiene and emergency preparedness have birthed opportunities for businesses to leverage and emerge as organizations people can trust.

KEY WORDS: COVID-19, CONSUMER BEHAVIOR, BUYING BEHAVIOR, ECONOMIC IMPACT, POST-PANDEMIC MARKETPLACE.

INTRODUCTION

The COVID-19 pandemic emerged from China's Wuhan district in late 2019 and followed varying patterns of emergence, infection and spread globally. As of September 29, 2020, it has reached 213 countries with more than 33 million confirmed cases and over 1 million deaths, challenging the health systems, economies and communities globally. Without any vaccines or appropriate medical interventions except symptomatic ones, most countries resorted to non-pharmaceutical interventions (NPIs), such as lockdowns, social distancing, closure of academic institutions and non-essential services/businesses, cancelling public gatherings and levying stringent travel bans (Stefan et al. 2020). These measures have given a new face to the global economy. The changes seen in the ways of living, thinking, buying and travelling are multi-faceted and the economic downturn is apparent in all sectors, but the service sector is most likely to suffer huge losses and job cuts. (McKinsey, 2020).

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Businesses and economic functions have been rendered inoperative in the wake of the preventive measures. The global recession is speculated to be more catastrophic than the Great Depression of 1929 and the Stock Market Crash of 2008-09. A 13-32% drop in worldwide trade is expected to occur in 2020 attributing to COVID-19 (Azevedo, 2020). India has to simultaneously deal with the COVID-19 crisis, its already staggering GDP growth and a 45-year unemployment high. FY-2019 observed a GDP growth of only 4.7%, lowest since 2013 and also a 5.2% fall in industrial output in the eight core output sectors, reportedly the worst in 14 years (Rastogi, 2020).

The outbreak has made the market tight-fisted and the consumers apprehensive (Carufel, 2020). Crumbling SCM practices and unplanned trade restrictions have caused business closures and jobs/salary cuts in all industries, which have altered consumers' personal financing behavior, entailing reduced consumption or spending, resulting into lesser flow of money into the economy. With the lockdown in force, the expenditure on fuel, dining and socializing has been reduced and the consumer spending behavior in the recently 'unlocked' states is cautious and household spending patterns have been majorly altered. The work-from-home culture has favored the use of online shopping platforms, widening the reach of e-commerce in both metros and non-metros. People have become increasingly aware of healthcare and hygiene practices, which have now become a force of habit.Worldwide cancellations of Mass Gatherings (MGs) since early March, have rendered the travel and tourism, entertainment and hospitality industries inoperative. Even though the restrictions are now being eased, the consumers are apprehensive. It has become crucial for retail businesses to re-evaluate and restructure their operations in accordance with the new market trends and constantly changing the what's and how's of buying behavior (Carufel, 2020).

The economic impact of the outbreak is becoming increasingly evident. The International Monetary Fund has estimated the global economy to shrink by around 3% in FY-2020 and a collapse is being feared even in the US, China, UK, Germany, France and Japan (Lawder, 2020 and Mahar, 2020). As per the Economic Survey of 2018-19, around 93% of the total workforce is informal and the Periodic Labor Force Survey (PLFS) 2017-18, reported that 6.1% Of India's Labor Force, which comprises around 17.8% of young people aged 15-29 years, is unemployed.

Acuite Ratings & Research estimates the lockdown to cost $4.5 billion to the Indian economy per day and Barclay's research pegs the output loss at $26 billion per week with zero GDP growth for India in FY 2020. Health security and global changes are determined by understanding how a disease outbreak impacts travel patterns and practices worldwide (Burkle, 2006). Travel parameters are integral determinants of disease epidemiology and disease surveillance mechanism development (Hon, 2013 & Khan, et al. 2009). The World Travel and Tourism Council estimates the impact of travel restrictions to be the most severe in the Asian continent. Globally, around 50 million jobs in this sector are at stake, and negative growth of 25% is being estimated (Faus, 2020).

The Indian Association of Tour Operators (IATO) estimates losses amounting up to INR 8500 crores in the hotel, travel and aviation sectors (Suri, 2020). Spending on road transport comprised more than 90% of the total travel expenditure and bus services were the most widely used in both rural and urban India, as reported in 2016 by the National Survey Sample Office (NSSO) (UTIP, 2020). The travel advisory for the pandemic suggests the use of only personal vehicles for all transportation purposes and avoiding all public transport (Saxena,2020). Consequently, a research conducted by IIT-Hyderabad and Bombay, reported that around 93% of respondents prefer personal vehicles over public transport (Pandey, 2020).

In the consumer goods market, reduced demand will be superseded by delayed demand. The companies and suppliers working on thin working-capital margins will be gravely impacted (Mckinsey, 2020). The virus spread, social distancing implications and increased awareness about COVID-19 have considerably impacted the social behavior of the people. Several prominent MGs have been cancelled or postponed along with various sports events including the Union of European Football Associations Euro 2020 football championship, the Formula 1 Grand Prix in China, the Six Nations rugby championship in Italy and Ireland and others. The Islamic pilgrimages of Umrah and Hajj in Saudi Arabia will be organized with stringent rules like tests for visitors, restrictions for people older than 65 and isolation of the visitors afterwards (McCloskey et al., 2020).

The 2009 outbreak of H1N1 virus, observed the emergence and application of the concept of 'mass gathering medicine'(Memish, 2019) owing to which, less than 100 pilgrims were diagnosed with Influenza A H1N1 09, including five fatalities, after Hajj ended on November 30, as the Saudi MoH reported (Haworth, Rashid, & Booy, 2020). Influenza outbreaks have been observed throughout the history of sporting and music events such as the 2002 Winter Olympics in Salt Lake City, UT, the USA, and music festivals in 2009 in Belgium, Serbia, and Hungary, and also at the World Youth Day in Sydney, NSW, Australia, in July 2008 (McCloskey, 2014). Kumbh Mela being one of the most significant religious mass gathering events (MGE) reportedly attracted a record of over 24 crore national and foreign visitors in 2019 (John, 2020).

Such mass gatherings have been observed as a public health threat in past instances, attributing to the spread of various respiratory, faeco-oral, vector-borne, zoonotic, gastrointestinal, genitourinary and other infections (Sridhar, Gautret and Brouqui, 2015). Several popular entertainment events like the Oscars, the Cannes movie festival and movie releases along with award shows have been either cancelled or postponed. The Events

and Entertainment Management Association (EEMA) is seeking aid to support the 60 million employees associated with it. The INR 183 billion Indian film industry is also estimated to face a loss of INR 200-250 crores over the next few months (Suri, 2020). Acuite Ratings and Research has attributed the disease to cause around 50 per cent reduction in multiplex footfalls in the upcoming months in the metros and Tier II cities (Shekhar, 2020).

Various studies have shown that densely populated fitness centers, gymnasiums and sports facilities could cause more infections. An instance of more than 100 people being infected with the virus occurred in a Zumba training workshop in South Korea (DeMarco, 2020). The Coronavirus has staggered the functioning of the fitness industry risking shutting down of smaller gyms and substantial revenue losses for larger chains leading to unemployment of thousands of fitness trainers and support staff (Economic Times , 2020a). In response to the virus, the household spending underwent a sharp increase initially in the retail segment, credit card purchases, food items and other FMCGs; later followed by a sharp decrease (Baker et al. 2020). Findings from another primary study reported that income and wealth losses averaging around $5,293 and $33,482, respectively were faced by almost half the respondents. Aggregate consumer spending observed a drop of 31 log percentage points with the travel and apparel sectors being hit the worst (Coibion, 2020).

The US observed a 5% hike in the personal savings rate in March which stood at 8% in February (Brancaccio, Wrenn, and Soderstrom, 2020). In the initial days of the crisis, a 50% increase in household spending was observed, with a 7.5% increase just in the grocery segment. A subsequent decline in spending followed the imposition of restrictions (Bhatia, 2020).The buying is more focused on essential goods and is mainly occurring through digital means and from local brands and providers (Accenture report, 2020). A study titled “Changes to the general lifestyle due to COVID-19 in selected countries 2020”, conducted on 2,137 respondents, was published by Alexander Kunst. The results of that study have been tabulated in Table 1 (Kunst, 2020).

Table 1. The results of the study published by Alexnader Kunst (2020)

QUESTIONS	GERMANY	UNITED KINGDOM	UNITED STATES
STAYED AT HOME MORE	71%	84%	77%
WASHED HANDS MORE	70%	75%	73%
APPLIED SOCIAL DISTANCING	61%	78%	69%
WEAR PROTECTIVE FACE MASKS OUTSIDE	52%	20%	66%
AVOIDED PUBLIC PLACES LIKE BARS AND RESTAURANTS	58%	67%	65%
GONE TO THE SHOPS LESS	59%	71%	62%
TRAVELLED LESS	51%	69%	56%
CANCELLED PLANS WITH FAMILY OR FRIENDS	54%	62%	53%
SHOPPED ONLINE MORE	30%	46%	52%
CLEANED YOUR HOUSE MORE	20%	40%	43%
AVOIDED PUBLIC TRANSPORT	50%	60%	36%
AVOIDED CERTAIN SHOPPING TIMES	37%	43%	35%
WORKED FROM HOME	22%	28%	30%
I HAVE NOT MADE ANY CHANGES TO MY LIFESTYLE	5%	2%	5%

Increased spending on survival goods and services has shifted the focus from non-essential and luxury goods (Singh, 2020). ‘Panic Buying’ induced because of increased consumer awareness about COVID-19 and a sense of fear regarding product shortages, was responsible for a hike in the sales of various healthcare and hygiene products like sanitizers, masks and household products like groceries, personal care products and others. As reported by Nielsen, a market research firm, the sales of hygiene products and medical-grade masks have increased by more than 300%, and some grocery products like shelf-stable milk and milk substitutes, with longer shelf lives, have reported a dollar growth of more than 300%. (Nielsen Report, 2020).

COVID-19 has proved to be even more disruptive than technology towards our working, shopping and communication mannerisms. A study conducted in the Chinese context by Mckinsey points towards increased consumer alignment with digital modes of shopping for household and personal care items. Other market surveys have revealed that around 3/4th of Internet users across China and South-east Asia have avoided crowded public places because of the virus scare (Reddy, 2020). E-Commerce platforms have seen a worldwide increase in their popularity and have considerably facilitated the entire shopping experience. The most massive increase in e-commerce has been reported in Vietnam, where 57% of consumers are switching to online purchases. India (55%), China (50%) and Italy (31%) have also seen widespread purchasing digitization, as recently reported by Ipsos (Clapp, 2020). These platforms have also been encountered by some safety concerns (Meyer, 2020).

However, the World Health Organization cleared up the confusion by deeming it safe to receive packages from locations where COVID-19 cases have been reported. The luxury goods industry has faced significant losses in the wake of the pandemic with companies like Vogue Business projecting losses amounting to around $10 billion for this segment in the FY 2020 (Biondi, 2020). The data collected from PropTiger.com shows a 26% decline in the housing sales in nine major Indian cities for the first quarter of 2020. Prospective real estate customers are highly likely to postpone their purchase decision as they await financial stability (Singh, 2020). Automobile companies like Maruti Suzuki, Honda, Toyota and Tata Motors, are estimating a rise in demand owing to the social distancing norms, fear of virus spread and reduced preference towards public transport (Economic times, 2020b).

The association of the virus with China has rendered the Indian marketplace hostile and Indian consumers unwelcoming towards Chinese products. In 2018-19, China exported well above 60% of electronic products and components and over 80% of antibiotics. Several Indian market segments are bound to feel the impact if Chinese goods are boycotted, putting thousands of jobs at stake and risking huge losses. (Martin, 2020).Assessing the purchase patterns of the consumers reveals that grocery is the most bought item accounting for 19% of the sales, followed by household products and personal care items at 13%, healthcare products at 12% and beauty products at 7%. 30% of respondents have reduced their frequency of shopping in stores (Carufel, 2020). As the pandemic advances, studies conducted in the Indian context have revealed that as high as 87.2% of Indians have shown a considerable increase in concern towards personal hygiene, attributing to behavioral changes. Health and hygiene brands have developed large-scale campaigns and strategies to spread awareness on the importance of cleanliness (Das, 2020).

METHODOLOGY

Research Question and Objectives: Idea of studying consumer behavior pattern, in the Covid19 era, was conceived after reviewing many research articles published on the impact of Covid19. Accordingly, the authors have structured the proposed study to understand in depth whether COVID-19 has impacted the consumer behavior and how it has influenced general lifestyle of the population. Also, the study proposes to find the association between COVID-19 and consumer behavior concerning certain sectors. To address the above research question, following research objectives were formulated: To investigate the effect of COVID-19 on the general lifestyles of the people.To examine the relationship between COVID-19 and Consumer Behavior pattern.

Hypothesis: Four hypotheses were also formulated to understand the impact of COVID-19 on the consumer behavior pattern. These hypotheses were developed with respect to buying behavior in certain specific sectors. H01: There is no relation between COVID-19 and Social Behavior.Ha_1: There is a relation between COVID-19 and Social Behavior.$H0_2$: There is no relation between COVID-19 and Travelling Behavior.Ha2: There is a relation between COVID-19 and Travelling Behavior.$H0_3$: There is no relation between COVID-19 and Spending Behavior. Ha_3: There is a relation between COVID-19 and Spending Behavior.$H0_4$: There is no relation between COVID-19 and Buying Behavior.Ha2: There is a relation between COVID-19 and Buying Behavior.

Study Design and Data Analysis plan: The proposed study is both descriptive and analytical in nature. Quantitative method is used in the study and primary data were collected through online structured questionnaire. Questionnaire was developed, having 29 questions, related to various aspects such as demography, social behavior, travelling behavior, buying behavior, income, savings and spending behavior of the respondents etc. Questionnaire was prepared using google forms and sent to nearly 500 individuals digitally. Samples were identified based on convenient and snowball sampling technique. Total 323 respondents sent their complete responses. Majority of the samples were from Delhi and NCR region.

Data were analyzed using Microsoft Excel, and the results were presented using absolute figures and percentages of total participants. MS-Excel spreadsheet was used to derive the pie charts and the bar graphs for analysis. Quantitative analysis is done using Microsoft Excel and SPSS-Version 24. The data were divided into four sections - Social Behavior, Travelling Behavior, Buying Behavior and Spending Behavior. The Chi-Square test has been used as a statistical tool to test the relationship and to draw some inferences at a 95% confidence interval on the data collected. A Chi-square test for independence compares two variables in a contingency table to see whether there is a statistically significant relationship between categorical variables.

It gives a p-value which indicates whether the test results are significant or not. A p-value that is less than or equal to the significance level (< 0.05) indicates that there is sufficient evidence to conclude that the observed distribution is not the same as the expected distribution. It can be concluded that a relationship exists between the categorical variables.

RESULTS AND DISCUSSION

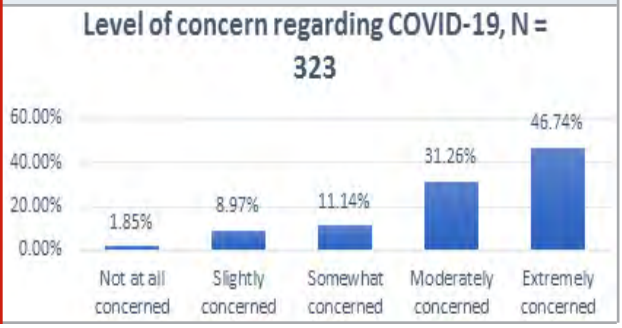
The demographic details of the participants have been given in the Table 2. The proportion of male respondents in the survey was found to be higher than females. Out of the total respondents, 73% were male. Majority of the participants were from the age group 19-30, i.e., 57% of the total respondents of the survey (Figure4.2). 95% of the participants were married. As far as the educational qualification is concerned, 44% of the respondents in the sample population were graduates, and 33% had doctorate degrees. 69% of the total respondents were employed in the private sector, and the participation of the respondents having monthly income 31000-50000

was higher than other income groups. Majority of respondents were found to show a high level of concern towards COVID-19. Around 48% of the survey population regarded the threat of the virus to be high (Figure 1).

Table 2. Demographic characteristics of participants in the study.

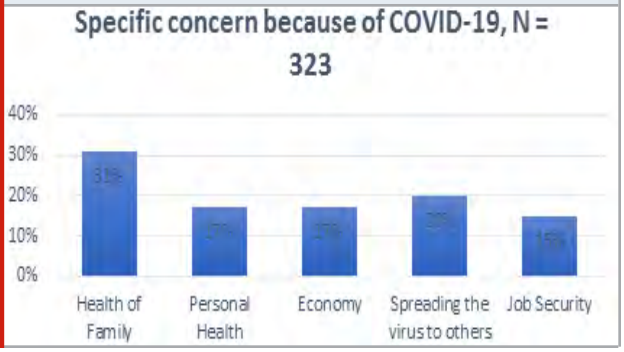
DEMOGRAPHIC INDICATOR	NUMBER	PERCENTAGE
GENDER		
MALE	237	73.4
FEMALE	86	26.6
AGE GROUP	1	0.3
19-30	183	56.7
31-45	118	36.5
46-59	17	5.3
MORE THAN 60 YEARS	4	1.2
MARITAL STATUS		
DIVORCED	1	0.3
MARRIED	147	45.5
UNMARRIED	175	54.2
EDUCATIONAL QUALIFICATION		
HIGHER SECONDARY	14	4.3
GRADUATION	102	31.6
POST-GRADUATION	177	54.8
DOCTORATE	30	9.3
OCCUPATION		
GOVERNMENT JOB	43	13.3
PRIVATE JOB	221	68.4
BUSINESS OWNER	43	13.3
HOUSEWIFE	16	5
MONTHLY INCOME DIVISION		
RS. 15000 AND LESS	55	17
RS. 16000-30000	69	22
RS. 31000-50000	116	36
RS. 51000-80000	62	19
MORE THAN RS. 80000	21	6

Figure 1: Level of concern regarding COVID-19, as reported by respondents



The respondents were specifically concerned about the health of their family members. The risk of spreading the virus to others and causing infections, was also one of the major concerns (Figure 2).

Figure 2: Specific concerns of the respondents regarding COVID-19



More than 50% of the sample population was of the view that in India, the disruption in normal lives of the people due to COVID-19 would take more than one year to recover and return to normal (Figure 3).

Figure 3: In how long life will return back to normal in India, as per respondents' perception

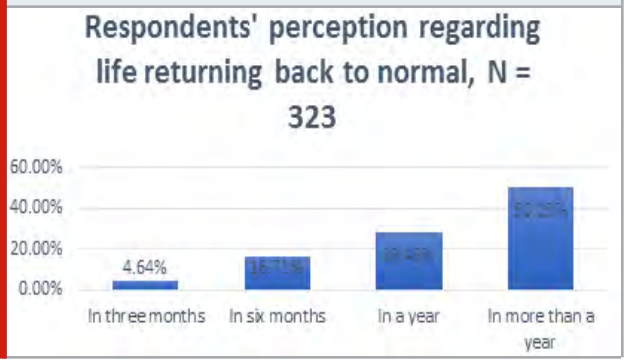
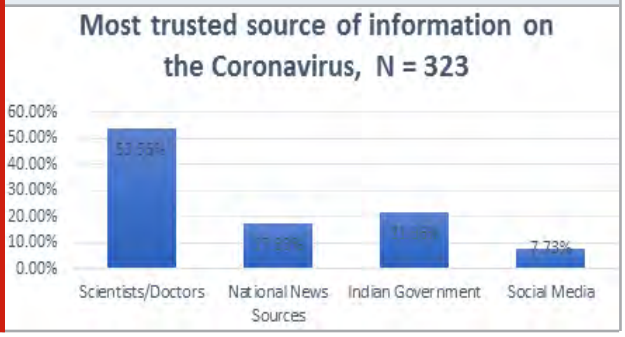


Figure 4: Various Sources of Information regarding COVID-19 as reported by respondents



The most trusted source of information about COVID-19 among the sample population were Scientists/Doctors, followed by the Indian government, then National news sources. Social media was considered the least trustable source of information (Figure 4). Table 3 describes the

impact of COVID-19 on the Social Behavior of the respondents where, 62% of the sample population was found to be staying at home and strongly unwilling to go out for public gatherings. Most of the respondents decreased their social interactions to protect themselves from COVID-19. Further, majority of the people surveyed would prefer not to go out for movies as well as for living concerts for some time even if COVID-19 is over.

Table 4 describes the impact of COVID-19 on the Travelling Behavior of the respondents Majority of the people have reduced their frequency of travelling and have only travelled sometimes during the outbreak. Further, the people were mostly found to be travelling for business purposes. Before COVID-19, the most preferred mode of the travel was private vehicles followed by public transport. For outstation travel, air transport was the second to last preferred mode of travel for the respondents . Even after COVID-19 majority of the people would be inclined towards using private vehicles, while the preference towards other modes has considerably decreased and rail transport was the least preferred.

Table 5 describes the impact of COVID-19 on the Spending Behavior of the respondents Almost 67% of the people said they feel that a recession or economic slowdown is inevitable (Figure 5). The uncertainty of the current situation has drastically altered the patterns of buying and spending among the consumers. 91% of the people have limited their expenditure due to COVID-19. Further, almost 61% of the population has increased their savings by up to 20% during the lockdown.

Figure 5: Respondents' perception regarding the occurrence of an economic slowdown.

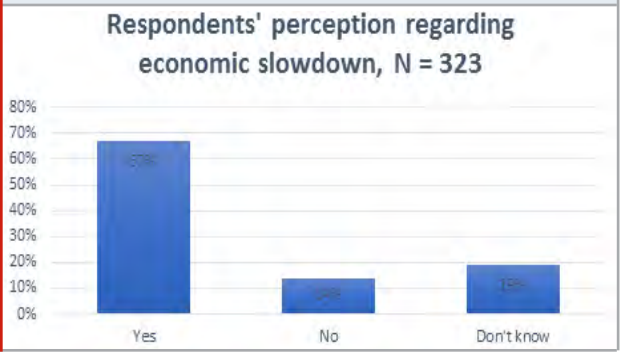
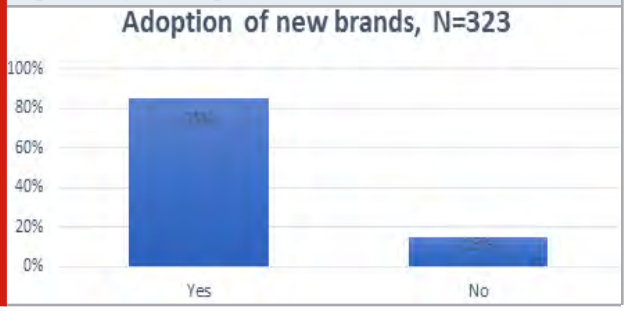


Table 6 describes the impact of COVID-19 on the Buying Behavior of the respondents Almost 81% of the population was affirmative regarding the impact of news about COVID-19 on the products they buy and admitted that it had impacted their buying behavior. The attitude of the people regarding Chinese-made products did not see much variations. Most of the people were not at all concerned about sourcing ingredients/parts from China while some of them were extremely concerned. 41% of the people were found to be likely to stop buying products from China. However, 37% of people did not share any opinion related to buying products from China. During the lockdown, the frequency of in-person shopping from the local shop decreased in around more than half of the population. Further, the research has found that the frequency of shopping from online platforms has decreased due to COVID-19. 61% of the population was concerned about the reliable fashion of delivery through online shopping. 85% of the population said that during the lockdown, they did not try new brands. (Figure 6).

Figure 6. Adoption of new brands during lockdown, as reported by the respondents



It is apparent from the Table 6 that people have bought grocery more than other products. People trying to stock up groceries due to the pandemic indicates 'panic-buying' which has led to an increase in the demand for these products as compared to the other categories, and also product shortages. The purchase of hand sanitizers saw an increase, followed by the purchase of disposable masks due to the pandemic. The majority of respondents, i.e., 158, said that they could not predict when they are going to buy big-ticket items while 88 respondents said that after a year, they would consider purchasing luxurious goods.

Hypotheses testing and Interpretation: The proposed study was conducted to know the behavioral impact of the pandemic on the people. And to study the relationship between identified variables, four hypotheses were made. These hypotheses were tested using Chi-square. Result of each hypothesis were described below H01: There is no relation between COVID-19 and Social Behavior.
Ha1: There is a relation between COVID-19 and Social Behavior.

To test the hypothesis cross tabulation is used between the following variables:

- Concern about Covid-19 and Social behavior.

It is apparent from Table 3 that there is a significant association between COVID-19 and the Social Behavior as the p-value is less than the significant value 0.05. 62% of the respondents are not willing at all to go out to public places due to COVID-19. The reason could also be associated with increased concern and awareness among the people about the virus. A total of 151 respondents are extremely concerned about the virus spread, and 33% of the sample population are doctorate holders, whereas 44% are graduates.

Table 3. The hypothesis ($H0_1$) was tested, and the results are given in.

SOCIAL BEHAVIOR STATEMENT	FREQUENCY (N)	PERCENTAGE (%)	P-VALUE*
ARE YOU STILL WILLING TO GO OUT FOR PUBLIC GATHERING EVENTS/ACTIVITIES?			
EXTREMELY WILLING	4	1.2	0.00
MODERATELY WILLING	7	2.2	
NOT AT ALL WILLING	203	62.8	
SLIGHTLY WILLING	80	24.8	
SOMEWHAT WILLING	29	9	
WHAT ACTIVITIES/EVENTS WILL YOU CUT BACK, WHICH INVOLVE PUBLIC GATHERINGS ONCE COVID-19 IS OVER?			
FITNESS CLASSES/ GYMNASIUM	55	17	0.00
SPORTING EVENTS	52	16	
LIVE CONCERT	71	22	
MOVIES	74	23	
RESTAURANTS/BARS	71	22	

Table 4. The above hypothesis (H02) was tested to know the impact of COVID-19 on travelling, and the results are encapsulated in.

TRAVELLING BEHAVIOR STATEMENT	FREQUENCY (N)	PERCENTAGE (%)	P-VALUE*
HOW FREQUENTLY DO YOU TRAVEL?			
ALWAYS	51	15.8	
OFTEN	72	22.2	
SOMETIMES	135	41.8	
RARELY	61	18.9	0.000
NEVER	4	1.2	
PURPOSE OF TRAVEL?			
BUSINESS	116	35.9	
HOLIDAY	61	18.9	0.000
MEETING RELATIVES	65	20.1	
OTHER	81	25.1	
WHAT WAS YOUR PREFERRED MODE OF TRAVEL (BEFORE SPREAD OF CORONAVIRUS/ BEFORE LOCKDOWN) *			
AIR	75	23.2	
RAIL	41	12.7	0.341
PUBLIC TRANSPORTATION (BUSES/METRO)	88	27.2	
OWN VEHICLE	119	36.8	
WHAT WILL BE YOUR PREFERRED MODE OF TRAVEL (AFTER CORONAVIRUS SPREAD/ AFTER LOCKDOWN) *			
AIR	56	17.3	
RAIL	15	4.6	
PUBLIC TRANSPORTATION (BUSES/METRO)	38	11.8	0.000
OWN VEHICLE	214	66.3	

So, it can be concluded that the respondents are well educated and are thus reluctant to join public gatherings knowing the consequences of the virus. The outcome of the results suggested to accept the alternate hypothesis, i.e., there is an association between the COVID-19 and Social Behavior and failed to accept the null hypothesis. H02: There is no relation between COVID-19 and Travelling Behavior.Ha2: There is a relation between COVID-19 and Travelling Behavior.

To test the hypothesis cross tabulation is used between the following variables:

- Concern about covid-19 and Travelling behavior.

Table 5. The hypothesis ($H0_3$) was tested, and results are précised in.

SPENDING BEHAVIOR			
STATEMENT	FREQUENCY (N)	PERCENTAGE (%)	P-VALUE*
HAVE YOU STARTED LIMITING YOUR SPENDING?			
YES	295	91.3	0.000
NO	28	8.7	
YOUR SAVING AS COMPARED WITH 2019 HAS INCREASED BY (%)			
0 – 20	206	63.8	
21 – 40	68	21.1	0.005
41 – 60	35	10.8	
61 – 80	12	3.7	
MORE THAN 81	2	0.6	

It is evident from the above table 4 that the frequency of travelers was affected significantly with a p-value less than 0.05. Moreover, the nation-wide ban on travel significantly impacted business-related travel. The preferred mode of transport after the lockdown changed and was statistically significant with a p-value less than 0.05 at CI of 95%, because people prefer to travel in their private vehicles rather than in public, air and rail transport. The researcher found that there was a significant impact on the travelling behavior of the people due to COVID-19. In this view, the null hypothesis was not accepted, i.e., there is no association between COVID-19 and Travelling Behavior.H03: There is no relation between COVID-19 and Spending Behavior.Ha3: There is a relation between COVID-19 and Spending Behavior. To test the hypothesis cross tabulation is used between the following variables: Concern about covid-19 and Spending behavior.

It is quite evident from the above results that COVID-19 is associated with the spending Behavior of the respondents with p-values less than 0.05. 36% of respondents of this survey belong to the income group ranging from 31,000 to 50,000. So, any change in the market will affect their buying and spending behavior. Consumers have started to limit their spending and have also increased their savings in contrast to 2019. The uncertainty accompanying the pandemic has urged people to spend less and save more. H04: There is no relation between COVID-19 and Buying Behavior.Ha4: There is a relation between COVID-19 and Buying Behavior. To test the hypothesis cross tabulation is used between the following variables: Concern about covid-19 and buying behavior.

It was found that the impact of news on the buying behavior of people was significant as the p-value 0.000 is less than the significant value of 0.05. Similarly, the frequency of shopping from local shops in person was significantly increased with a p-value less than 0.05 due to all the malls and big grocery stores being closed during the lockdown. Further, there was a significant decline in online shopping with a p-value less than 0.05 due to the lockdown and people were also reluctant to buy things online because they were not sure about whether or not the delivery personnel were following the suggested health and hygiene practices.

There was a statistically significant increase in the purchase of products and grocery topped the list among all the categories. Hygiene practices were more discussed due to COVID-19 and to prevent its spread. As it is evident from the Chi-square results, there is a significant increase in the purchase of hygiene products with a p-value less than 0.05. The majority of the respondents belong to the middle-income group, which is also an influencing factor for buying behavior. The results of the data are in support of the alternate hypothesis (Ha4), so the researcher failed to accept the null hypothesis, i.e., there is no association between the COVID-19 and Buying Behavior.

Whenever or wherever a disease outbreak has occurred, it has led to chaos and adverse economic repercussions (Jarus, 2020). The occurrence and spread of disease, whether the Spanish Flu of 1918 or the HIV/AIDS pandemic of the 1980s, have always led to economic downfall worldwide. The COVID-19 pandemic is no

different. The negative impact on the population and widespread economic ramifications were the inevitable results of the enforcement of lockdowns and travel restrictions. Every sector was impacted in some way. People lost financial stability, job security and their savings were gravely impacted. The dynamics of consumer buying behavior, lifestyle and travel as well as social behavior have been altered drastically.

Table 6. The above hypothesis (H04) was tested, and the results are summarized in.

BUYING BEHAVIOR STATEMENT	FREQUENCY (N)	PERCENTAGE (%)	P-VALUE*
IS THE NEWS ABOUT CORONAVIRUS IS IMPACTING WHAT PRODUCTS YOU ARE PURCHASING?			
YES	262	81.1	0.000
NO	61	18.9	
ARE YOU CONCERNED ABOUT INDIAN/ FOREIGN BRANDS SOURCING INGREDIENTS/PARTS FROM CHINA?			
NOT AT ALL CONCERNED	79	24.4	
SLIGHTLY CONCERNED	72	22.3	
SOMEWHAT CONCERNED	59	18.3	0.163
MODERATELY CONCERNED	47	14.6	
EXTREMELY CONCERNED	66	20.4	
WILL, YOU STOP BUYING PRODUCTS FROM CHINA?			
YES	133	41.2	
NO	71	22.0	0.903
CAN'T SAY	119	36.8	
DURING THE LOCKDOWN YOUR FREQUENCY OF IN-PERSON SHOPPING FROM THE LOCAL SHOP HAS			
INCREASED	173	53.6	
DECREASED	116	35.9	0.000
NOT CHANGED	34	10.5	
DURING THE LOCKDOWN YOUR FREQUENCY OF SHOPPING FROM ONLINE PLATFORMS HAS			
INCREASED	71	22.0	
DECREASED	182	56.3	0.000
NOT CHANGED	70	21.7	
ARE YOU CONCERNED ABOUT THE RELIABLE FASHION OF DELIVERY THROUGH ONLINE SHOPPING			
YES	196	60.7	0.000
NO	127	39.3	
WHAT PRODUCTS ARE YOU BUYING MORE? (CAN SELECT MORE THAN ONE OPTION)			
GROCERY	135	42	0.000
HOUSEHOLD PRODUCTS	81	25	
PERSONAL CARE PRODUCTS	39	12	
HEALTHCARE PRODUCTS (OTC MEDICINES ETC.)	68	21	
HOW MANY ITEMS HAD YOU PURCHASED SINCE YOU CAME TO KNOW ABOUT COVID-19? (CAN TICK MORE THAN ONE OPTION)			

Table 6 Continue

HAND SANITIZER	97	30	
DISINFECTANT WIPES	35	11	0.000
ANTIBACTERIAL SOAP	55	17	
DISPOSABLE FACE MASKS	84	26	
DISPOSABLE GLOVES	52	16	
WHEN WOULD YOU CONSIDER PURCHASING BIG-TICKET ITEMS (HOMES, CARS, TRIPS, LUXURY GOODS)?			
0 – 3 MONTHS	16	5.0	
4 – 6 MONTHS	23	7.1	0.000
7 – 12 MONTHS	38	11.8	
AFTER A YEAR	88	27.2	
CAN'T PREDICT	158	48.9	

An analysis of the collected data shows that COVID-19 has changed the world as we know it. Various sectors targeted in the study are significantly associated with the virus and maintain a direct correlation with the overall behavior of the people. Majority of them are avoiding social gatherings, and the frequency of travelling has also decreased. The fear of recession and economic downfall has significantly affected the spending behavior of the respondents, with an increased focus on savings and limited spending. The demand for many luxurious goods and services have taken maximum hit and people are not willing to buy things that are not in absolute necessity.

This study has aided in spotting various business opportunities that likely to change for short term or long-term post COVID-19. The behavioral changes that affected demand creation were studied in the proposed study. The opportunity to turn this change into a profit-making venture for healthcare and hygiene products is enormous, with hiked demand estimated to be long term or even permanent. Results of the study, to a larger extent supported the outcome of literature studied for the paper. Consumers resorted to bulk buying or panic buying fueled by their fear of the lack of resources and shortcomings of the domestic supply chain, creating a new direct link between consumers and wholesale channels.

With an increasing number of shoppers becoming interested in knowing the source of the products, they are buying it is safe to assume that the crisis may ignite a long-term trend in the popularity of domestically made products, leading to a resurgence for 'Made in India' products. The need for 'germ-free' homes and a mandate for 'safe' public places, will lead to budding 'Sanitizing' businesses pan India. Companies or brands associated with in-home entertainment have seen a massive demand-surge during the pandemic. An increase in domestic tourism and 'rental car boom' is likely, owing to air travel restrictions and lack of confidence in public transport. The aviation and hospitality sectors will need to reposition their businesses to cater to the needs of a changing market.In times of crisis, consumers are often known to gravitate towards traditional ways of buying goods and opting for services, perhaps driven by the perception of being in control and limiting their spending.

The current spike in e-commerce and online services may be short-lived, and the post-pandemic market could see an increased inclination towards 'offline' shopping. Supply chain transparency is now of paramount importance pertaining to the fragility of the global supply chain made apparent by COVID-19. It is integral for companies to improve their SCM by focusing extensively on various data-driven tools, emergency-proofing and risk-preparedness. Consumers are more likely to lean back on the brands they trust, and newer brands might be faced with apprehension in a tight-fisted market. In such situations, brands should morph into reliable sources of information to the consumers, along with initiating modifications in their business models and strategies. The market demand needs to be deduced by listening to the consumers and finding new solutions that they can trust.

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Analysis of Anti-Mosquito and Antimicrobial Activities of The Extract of *Nigella sativa* (Black seeds)

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ABSTRACT

Nigella sativa (Black seed) is an annual flowering plant, belongs to the Ranunculaceae which has tremendous usage in medicine. In the treatment of digestive tract disorders, including gas, colic, diarrhea, dysentery, constipation, and hemorrhoids, the black seed is used. It is also used for asthma, allergies, cough, bronchitis, emphysema and other respiratory conditions. The seeds (50 g) were crushed, extracted with 70% methanol and filtered through filter paper. Antibacterial activity of the extract was assessed using the spread plate method against the *Candida albicans*. Different concentration of the extract i.e., 20, 40, 60, 80, 100% made and applied against mosquito larvae to determine the larvicidal activity of the extract. The results showed that the extract exhibited significant antimicrobial activity against *C. albicans* on the culture plate as compared to control. Different dilution showed larvicidal activity in a dose-dependent manner; however, 50% of larvicidal was noticed in all dilutions after 24 h. However, maximum activity was recorded in 60, 80, and 100% dilutions after 72 h. The results showed that the extract was extremely effective against the microbes even at the concentration of 50%. Similarly, the extract was very effective against microbes and could be used as an antimicrobial agent.

KEY WORDS: NIGELLA SATIVA, BACK SEEDS, ANTI-MOSQUITOES, LARVICIDAL, ANTIMICROBIAL.

INTRODUCTION

Nigera sativa (Black seed) is a herbaceous plant, grows annually; however, is also cultivated in different parts of the world, including Asia, Africa, and America. Black seeds are derived from the common fennel flower plant (*Nigella sativa*) of the Kennebug family (*Ranuncula family*) (Ahmad et al., 2021; Soltane et al., 2021). Black seeds have long been known and used for medicines (Ali and Meitei, 2011). The black seeds have been also used and recommended for the treatment of minor disorders such as skin conditions and allergies (Prashar et al., 2004). Prophet Muhammad (PBUH) illumined in the medicinal importance of the black seed. A large number of studies have been reported in a detailed review on the medicinal use of super foods of plant origin, based on the recommendations of Prophet Muhammad, (PBUH) by Ali et al., (2018).

In recent years, research in the field of agriculture has focused on the economy of plants influenced by selective minor elements. Secondary metabolites from plant extract acts as reducing and stabilizing agents to produce new metallic nanoparticles through bio-reduction reaction (Jang et al., 2020). Comparatively, using a biological method for synthesizing nanoparticles is superior to a

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non-biological (chemical, physical) one, the former is inexpensive easy, and eco-friendly while the latter is highly toxic and hazardous for living organisms, (Bharali et al., 2013 Ullah et al 2020).

The mosquitoes are the carrier of several diseases known as vector-borne diseases. The diseases are including yellow fever, malaria, dengue fever, and many others. Black seeds have reported being toxic against the larvae as well as adults of the *T. castaneum* even at extremely lower concentrations (Dagli et al., 2015). Moreover, plants source are a potent source for treating several diseases such as Cancer, Malaria, Human immunodeficiency virus (HIV), Hepatitis, and many other serious ailments (Dris et al., 2017). it has been mentioned in one study about the punctuality and application of plant extract in different fields including medicine, nutrition, and energy (Enan, 2005).

On the other side, the application for curing different bacterial and viral diseases in pharmacological industries is a remarkable advantage (Sabbah et al., 2020; Helaly et al., 2020). Several studies have demonstrated the antibacterial activity of black seed oil against a variety of microorganisms (Ait Said et al., 2015). This effect is shown against *Salmonella* spp, *E. coli, Shigellas* pp. The biological method is cost-effective than others due to the abundant availability of biological entities easy procedure, hence highly explored for syntheses of nanoparticles (Zuzarte et al., 2012). The present study was designed to determine the larvicidal and antimicrobial activities of the extract of black seeds.

MATERIAL AND METHODS

Methanolic extraction: The black seeds were rinsed in distilled water for removing surface dust particles. After washing the seeds were kept under sunlight for drying. The seeds were crushed with the help of liquid nitrogen and mortar. The powered seeds were transferred to the flask containing 70% methanol. After homogenization, the mixture was incubated on a magnetic stirrer at room temperature for two days. The mixture was filtered by using Whatman filter papers no.1. Furthermore, the filtrate was dried and was dissolved in deionized distilled water.

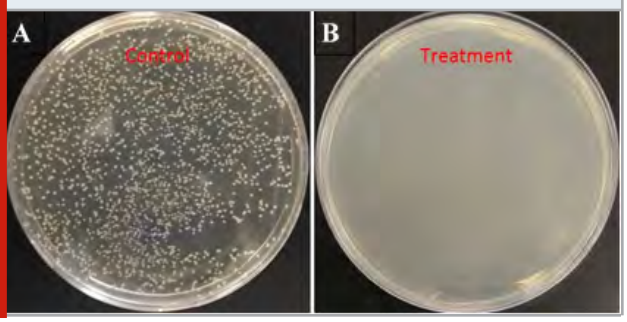
Antimicrobial activity of the extract: The fungicidal activity of the biogenic silver nanoparticles was assessed against the infectious fungi *Candida albicans*. This activity was determined using the colony-forming method. The *C. albicans* was cultured on PDA (potato dextrose agar) at 37°C, for 48 hours. Potato dextrose agar media were prepared and autoclaved. The autoclave media was transfer to the Petri plates and wait until solidification. The extract was applied on the plates followed by the transfer of *C. albicans* culture. The plates were incubated for 48 hours at 37°C.

Larvicidal Bioassay of the extract: The larvae of the mosquito were obtained from the Mosquito Research Laboratory, King Abdulaziz University Saudi Arabia. The larvae were reared in containers (25 × 35 × 6 cm) filled with up to the marked water supplemented with artificial food. The colony was maintained under the following conditions: 27°C ± 2°C, 70% ± 5% relative humidity. Different dilutions of an extract of black seed i.e., 20, 40, 60, 80, 100% (w/v) were used to assess the larvicidal activities. The larvae were transferred to each well of the six-well culture plates. The larvae were given standard larval food and the temperature was kept 28 ± 2°C. After 24 h of exposure, percentage mortality was noted. The experiment was repeated three times.

RESULT AND DISCUSSION

Antimicrobial activity of the extract: The spread plate method was used to assess the antimicrobial activity of the extract against *C. albicans*. The spread plate method is widely employed to check the susceptibility of chemical components of extracts against the microbes. The results revealed that the extract consisted of metabolites, responsible for antimicrobial activity against *C. albicans* (Fig. 1A and 1B). The surface of the plates i.e., control and treated showed the efficacy of the extract. No colony was observed on the plate treated with the extract. Hazardous effects associated with the usage of synthetic chemicals, it is important to introduce natural products, of plant origin. The plant origin compounds will reduce the harmful effects in the environment. The results of our study revealed that the extract showed 100% fungicidal activity against *C. albicans*. It has been reported that the aqueous extract of *N. sativa* exhibits an inhibitory effect against *C. albicans* (Ait Said et al., 2015). In the other study, Al-Jabra et al. reported that the ether extract of *N. sativa* seed showed inhibitory effects against *T. interdigitale*, *T. rebrand* (Zuzarte et al., 2012).

Figure 1: Antimicrobial activity of the extract of black seed against *Candida albicans* grown on potato dextrose Agar. (A) *Candida albicans* grown without application of extract represents control. (B) *Candida albicans* has grown with the application of extract.

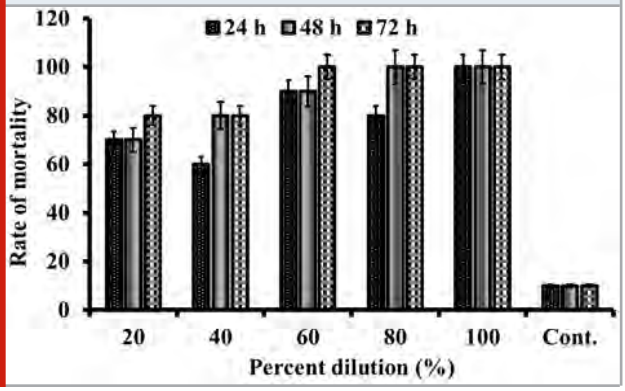


Larvicidal activity of the extract: The extract showed great larvicidal activity against the larvae of the mosquitoes. The rate of mortality shown in results (Fig. 2) represented the range of mortality in the mosquito larvae and revealed the insecticidal activity of ethanol extracts. The per cent mortality of the extract was directly related to the concentration of the extract as well as the time of exposure. The results exhibited that the maximum larvicidal activity was recorded by 60,

80, and 100% dilutions after 72 h of exposure. However, more than 50% mortality was recorded in all dilutions even after 24 h. In addition, the extract showed great larvicidal activity against the larvae of the mosquitoes in a dose-dependent manner.

The percent mortality of the extract was directly related to the concentration of the extract as well as the time of exposure (Abutaha et al., 2018). It has been reported that extracts of plants such as *Ageratum conyzoides* and *Cassia sophera* induced mortality in mosquitoes (Ghosh et al., 2012). Moreover, a *Mentha piperita* extract resulted in maximum insecticidal activity against *B. brassicas* with increased concentration and time exposure (Mersha et al., 2014). Similarly, essential oil from *Cinnamomum zeylannicum* was found to be effective against *B. brassicae*. The results showed that the extract was extremely effective against mosquitos and also showed antimicrobial activities.

Figure 2: Larvicidal activities of different dilutions of the extract of black seeds.



CONCLUSION

The results showed that the extract was extremely effective against mosquitos and also showed antimicrobial activities. The methanolic extract was dried and dissolved in different concentrations in water e.g., 20, 40, 60, 80, and 100%. All the dilutions were effective and killed the mosquito at their larval stages even within 24 h. similarly, the extract showed great antimicrobial activities against *C. albicans*.

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Corona Virus -19 Lockdown and its Impact on the Educational Status of Undergraduate Students of South Bengal, India

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ABSTRACT

The pandemic of coronavirus (COVID-19) has directly or indirectly affected human civilization and has recently given a tremendous challenge to humanity for leading a normal life. Academic and research activities of students have been very much hampered during this period. Education is an important vehicle through which students can analyze while making life decisions. Through proper education, they will get important knowledge, including basic facts, job skills, and cultural norms values. Education prepares our students to think properly about communication, social interaction, ethics, environment and sustainability. Although online classes are being organized by many educational institutions, rural students face a lot of problems in attending such classes. So, to access the impact of COVID-19 on the educational status of the undergraduate students, an online survey was done in the form of an online Google form and it was circulated to students of Bankura and Purulia district through several Facebook groups as well as WhatsApp and Telegram contacts with the help of college teachers. The Google form was designed in such a way, that students can respond only one time by using one email and it can be generated using one device at a time. A total of 170 students actively participated in this online survey platform. 37% of respondents said that their average study time decreases during these days. More than 40% of online classes missed by 21% of students and 20-40% of online classes missed by 38% of students due to poor network connectivity in their locality. The number of missed online classes increased during the afternoon and evening because of internet connectivity problems. Sometimes students faced headaches and eye irritations due to the excessive use of smartphones, so few classes were also missed for these reasons. In view of these issues some initiatives are urgently needed to reduce the psychological stress of students during the COVID-19 lockdown situation.

KEY WORDS: PANDEMIC; ON-LINE; GOOGLE; NETWORK; STUDENT; PSYCHOLOGICAL STRESS.

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INTRODUCTION

Human civilization has now faced a tremendous challenge from the novel severe acute respiratory syndrome coronavirus (SARS-Cov-2) in different countries. On January 30, 2020, the World Health Organization (WHO) declared Corona Virus Disease (COVID- 19) outbreak as an international public health emergency. Corona virus circulates in some wild animals like bats and transmitted to humans. It can cause severe respiratory symptoms along with symptoms of common cold and fever(Zhu

et al.,2020). The disease was first reported in China in December 2019 and now rapidly spread in many countries from Asia, Europe, and Africa(Jones,2020).The disease has now spread to 217countries and territories around the world and the total number of confirmed infected people is 42,055,863 people throughout the globe. In India, the disease was first reported on 30 January 2020 in Kerala from a student who returned from Wuhan, China. Now the total number of confirmed infected people is 7814682 till now across India (24th October, 2020) (WHO, COVID-19 Database). In West Bengal, many districts are also affected by this virus. The main problem of this outbreak is there are no specific treatments for these viruses to date. However, only one can reduce the chances of infection by only maintaining basic personal hygiene and social distancing from infected persons (Rubin and Wessely, 2020; Pulla, 2020).

Most of the people are now primarily restricted to their homes, owing to nationwide lockdowns and home-confinement strategies implemented in the majority of the COVID-19-hit countries to prevent the spread of the disease among communities (Rubin and Wessely,2020; Pulla,2020).This outbreak directly has hampered the normal lifestyle of people; many persons have lost their jobs and are facing tremendous mental stress. Due to the global outbreak of corona virus disease (COVID-19), the psychological issues have rapidly increased the public health burden of various persons (Totaleset al., 2020). Similarly, in this situation, the daily schedules of school, college, and university students are very much hampered and delays in academic activities mostly affected due to the closure of academic institutions (Cao et al., 2020). For college students, heightened levels of psychological distress and downstream negative academic consequences are prevalent under normal circumstances. During this adverse situation, education institutions shifted their classroom teaching to an online mode of teaching which would be expected to create academic stress for students. This creates a lot of problems in India because as per the 2017-18 national sample survey report only 24% of Indian household share internet facilities for education (Cao et al., 2020).

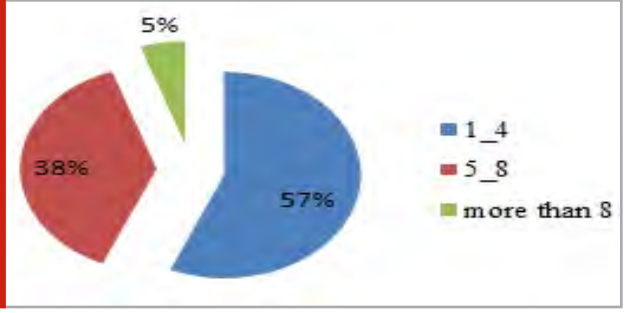
66% of the Indian population lives in rural areas where network connectivity is poor and only a little over 15% of rural households have access to internet services. In urban areas, only 42% of the population have access to internet services. More than 40% household has access to the internet in the states like Punjab, Kerala, Delhi, Uttarakhand and Himachal Pradesh whereas the proportion is less than 20% in the states of West Bengal, Bihar, Odisha, Assam, Andhra Pradesh, Jharkhand (Mukherjee and Roy,2020). During this online mode of teaching, students may experience reduced motivation toward studies, increased pressures for self-learning, changes in daily routines, and higher rates of dropout occur during this pandemic situation(Grubicet al.,2020).

In south Bengal regions, good percentages of student belonging to SC/ST/OBC and minority categories and many of them are even first-generation learners. This region is economically backward and no big industries are established in these regions. Students of this region are very talented, hard-working and procure top positions in board examinations in different University examinations in comparison to other districts of West Bengal (Mukherjee and Roy, 2020).In this background, the present study was planned online to evaluate the consequences and impact of Covid-19 lockdown among undergraduate students of South Bengal, India. During the online survey mainly students from two districts namely Bankura and Purulia were targeted. Through this study, we tried to understand their sociological status, mental health condition, pros and cons of the online mode of teaching during the lockdown situation.

MATERIAL AND METHODS

For understanding the impact of Covid-19 lockdown among undergraduate students of South Bengal, India, an online survey was conducted from 11th August to 24th August 2020. The survey was done in the form of an online Google form and it was circulated to students of Bankura and Purulia district through several Facebook groups as well as WhatsApp and Telegram contacts with the help of college teachers. The Google form was designed in such a way, that students can respond only one time by using one email and it can be generated using one device at a time. Due to the online mode of the survey, we specified our eligibility criteria (only students of South Bengal, India) to restrict its spread to a specific geography. The self-administered a questionnaire consisting of basic information about the students' names, age, gender, and the semester of study. Similarly, other questions were set to evaluate the effect of lockdown on their study, health as well as to understand the socio-demographic standards and psychological effects. The questionnaire was also included related to COVID-19 disease, its health hygiene, and information gathering related to this disease during the lockdown.

Figure 1: Number of family members in each student

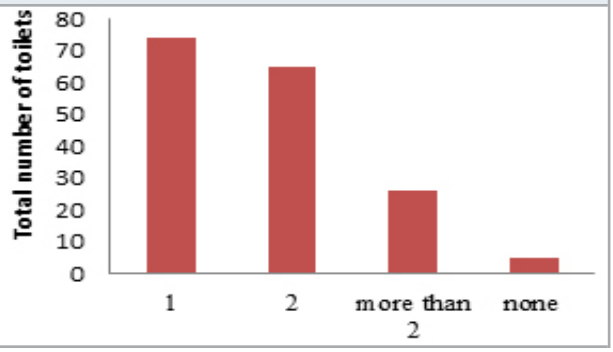


RESULTS AND DISCUSSION

A total of 170 responses were obtained from undergraduate students in the study period through the Google form survey platform. The data collected from respondents were analyzed and presented in Figures. Among 170 respondents, 26% of responses received from final six-

semester students, and only 9% of responses received from new second-semester students. Other semester students moderately participated in this online survey. The data are shown in Figure I revealed that 57% of students belonged to nuclear families and only 5% of student have belonged from joint family. The data shown in Figure II revealed that the majority of the respondents (44%) had only one toilet in their house and only 3% of students had no toilet, and family members still used open space for the toilet (Mukherjee and Roy, 2020).

Figure 2: Number of the toilet in each student family



The data shown in Figure III revealed that the average time of use of smart phones increased in the majority of the respondent's during the COVID-19 lockdown period. Out of 170 respondents, 70 students regularly used smart phone more than 4 hours in social media and 74 students used 2-4 hours in social media and it will directly affect the academic performance of students. Most respondents admitted that their study time decreased during the lockdown period. According to the internet and mobile association of India report 2019, 67% of men had access to the internet whereas in women it is only about 33%. This type of gender-wise disparity is very much prominent in villages areas where 72% of access done by men and 28% by women. But due to pandemic situation students are forced to attend their online classes so the disparity reduced from percentage proportion of men: women of72:28(all India level) to 58:42 (approx.) in south Bengal regions (Mukherjee and Roy, 2020).

The data shown in Figure IV revealed that during lockdown majority of the respondents (47%) received information related to COVID 19 and its precautions through social media like WhatsApp and Facebook. 13% from television, 13% from the governmental announcement, and 8 % from the newspaper. According to Dubey et al.,(2020)social media must be used for good purposes to educate people about symptoms and transmission COVID -19 diseases during this pandemic situation. At the same time implementation of strict governmental laws and legislation is needed to stop the spreading of fake news, rumours, disinformation, and misinformation (Dubeyet al.,2020).Similarly, Guptaet al., (2020) documented that the sleep pattern of respondents was very much influenced by the lockdown. Shift to a later bedtime, delayed sleep onset, daytime napping increased are very common among many people's (Gupta et al., 2020). Similarly, more than 40% of people faced anxiety and depression during this lockdown and pandemic situation (Grover et al., 2020).

Figure 3: Average time of Smartphone used during lockdown

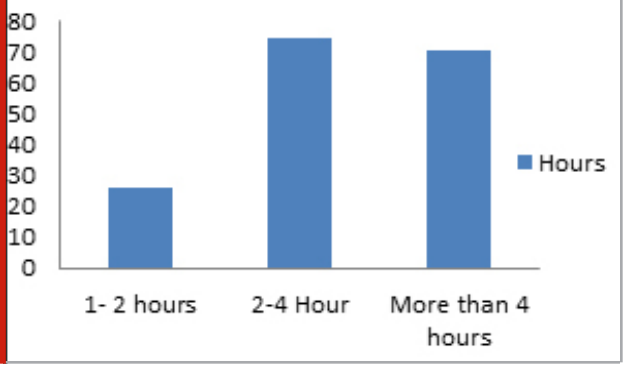


Figure 4: Awareness creates against COVID-19

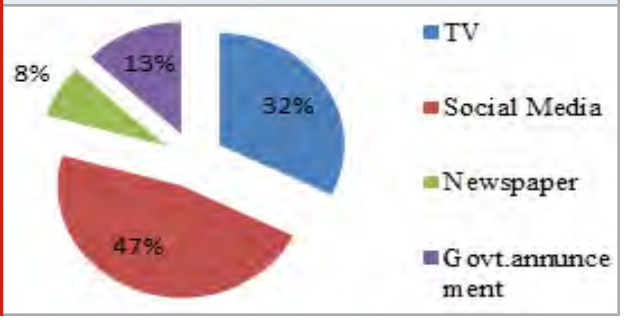


Figure 5: Types of ration card among student family

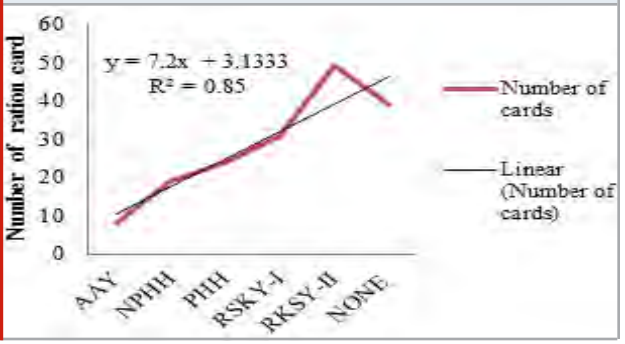
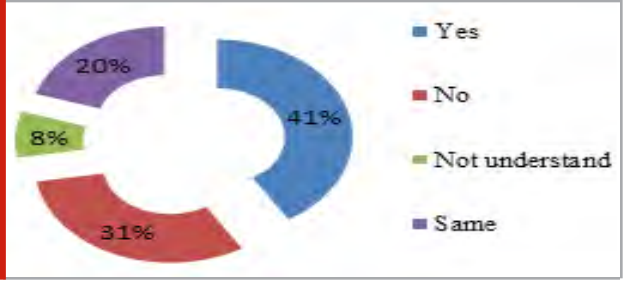


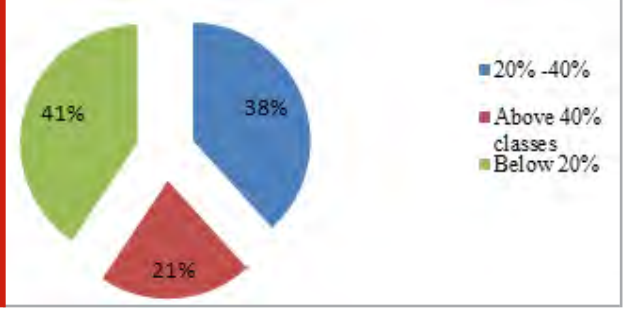
Figure 6: Bodyweight change during the lockdown



The data shown in Figure V revealed that most of the respondents have RKSY types of ration card and few students came from economically backward family. Till today few families do not have any ration cards. So, students from economically backward suffer a lot to attend the online classes. Similarly, the data shown in Figure VI reveals that 41% respondent gains their body weight during lockdown period because of leading sedentary life. The data were shown in Figure VII revealed that among all, 21% of students missed more than 40% of online classes, 38% missed 20-40% of online classes due to poor network connectivity in their locality. The number of online classes missed increases during the afternoon and evening because of connectivity problems as well as the exhaust of net data pack due to spending more time with smart phones during the daytime by students. Another survey also supported that due to closure of the educational institution, loss of classes and restricted social connections worsened pre-existing mental health conditions of 83% of young respondents(Youngminds,2020).

Dangi and George (2020) also showed that 76.44% of students suffered severe anxiety and 23.66% of students were having moderate anxiety in this lockdown period in their study area (Dangi and Mathew,2020). Sometimes students face headaches and eye irritation due to the use of excess time on smart phones so few classes also missed due to this reason. During the lockdown period, students were compelled to stay at home so during leisure time students learn many things. Girl students learned to prepare many food items from the internet, some prepare beautiful drawings as well as stitch work. Boys also spend time with physical exercise and nature study of their surroundings by using smart phones. Few students actively took part in many international and national online webinars which allow them the opportunity to listen to many things from reputed resource persons from home during this lockdown period (Dangi and Mathew, 2020).

Figure 7: Missed online classes due to poor internet connectivity



CONCLUSION

In the last two decades, mankind faced five pandemics like SARS (2002), Swine flu (2009), MERS (2012), Ebola (2014), and COVID-19 (2019). COVID-19 produces long-lasting effects on the various strata of people in society including students. The current pandemic situation does not obey the geographical boundary, race, religion, caste, and language. It creates psychological, social, economic, religious problems as well as depression and anxiety among people all over the world. It also produces immense agony among student fraternity who are already burdened with their semester and competitive exams, research and academic activities. This study showed that students are trying to adapt the new mode of online teaching but 21% of students missed more than 40% of online classes due to poor network connectivity in their locality. This creates a lot of mental anxiety among students along with loss of study time and restricted social connections. So, to reduce mental weakness and anxiety, educational institutions should have to conduct motivational sessions and set up one mental health helpline number. Arrangement of awareness programmes at both personal and community levels is urgently needed to reduce the psychological stress of students during the COVID-19 lockdown situation.

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Oxidative Degradation of Biodiesel: Modelling of Different Paths and Effect of Antioxidants on two Mechanisms

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ABSTRACT

Since past many years, biodiesel has been considered as a locally delivered, clean-consuming, inexhaustible substitute for oil diesel. Utilizing biodiesel as a vehicle fuel expands energy security since it contains large amount of methyl esters in the form of especially poly-unsaturated methyl esters. Due to presence of low oxidative stability, they can get easily oxidised and their long storage results into degradation. In order to control this oxidation process, antioxidants are used. Various studies have been performed concentrating upon the oxidative stability but very few focussed upon the mechanisms involved in oxidative degradation and the effect of antioxidants on reactions. The present study models the two different pathways of oxidative degradation of biodiesel for 5 years using the MATLAB 2020 and study of consumption of antioxidants to control the reaction mechanism has taken place. Comparison has been done between the oxidation of biodiesel in the absence and presence of antioxidant via the explanation of two different mechanisms. Change in concentration and consumption of RH (biodiesel organic matter) and antioxidant has been studied during the two different paths of oxidation. It has been concluded that antioxidant has affected the path one process more as compared to the path two process during the degradation mechanism of biodiesel in 5 years. Whereas consumption of antioxidant in path one is only approximate 3% as compared to 99% in path two. Therefore, oxidative degradation carried from path one can be controlled with lower amount of antioxidant whereas no such effect of antioxidant takes place during reactions of path two even after its complete consumption.

KEY WORDS: ANTIOXIDANTS, BIODIESEL, OXIDATION, METHYL-ESTERS, DEGRADATION.

INTRODUCTION

Since, many years, research has been performed for variety of energy resources which is nearly as old as the man's need of energy. Energy has now become the most important part to be considered for global economic growth. To meet the energy requirements, the use of fossil fuels is highly discouraged due to its contribution in greenhouse emission gas to environment, increasing instability in supply and rising production cost (Bannister et al., 2011). Similarly, now-a-days, petroleum reserves are diminishing at a high rate and petroleum fuelled engines are affecting the environment in negative manner, due to which, importance of alternative fuels is increasing. Therefore, urgent need has raised to replace fossil fuels to fulfil the demand of the world. Biofuels said to have more focal points over petroleum derivatives and other sustainable wellspring of energy since it lessens reliance on unfamiliar oil, it is delivered locally subsequently

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making work and elevating the economy, and has zero or unimportant outflows (Muhammad et al., 2018; Kåberger, 2018).

Hereby, biodiesel has become as an attractive and essential energy resource. Biodiesel contains large percentage of unsaturated methyl esters, which are especially in the form of poly-unsaturated methyl esters. They have comparatively lower oxidation stability than petroleum diesel. When the biodiesel is stored over time, their oxidative stability becomes a matter of concern. The storage problems that are merely caused by storage conditions are exposure to air, light, temperature as well as presence of metals with catalytic effect on oxidation (Sharma et-al, 2009; Karavalakis et al., 2010). To check the biodiesel quality control, oxidation stability is used as a parameter. The oxidation degradation process of biodiesel depends upon the factors such as fatty acids used in production, humidity, temperature and light. The biodiesel deterioration during operation has become the concern for many industries in recent years (Maia et al., 2011; Delgado et al., 2019).

Products formed during oxidation, not only affects the life in storage but also cause fuel filters clogging. To improve the oxidation stability, antioxidants are used by removing the free radical formed at initiation stage. The use of antioxidants helps in delaying of onset of oxidation with free radicals to form stable compounds which do not contribute to oxidation chain reactions. Natural antioxidants like tocopherols, sterols etc are found in vegetable oils, which are strongly affected by production and refining of biodiesel and parent oils respectively. Antioxidants stabilized the fatty peroxy radicals and hereby, the chain reaction gets stopped. Antioxidant do not inhibit but retards the polymerisation. Indicators like formation of colour, presence of soluble gums and insoluble in the fuel helps in stability. (Razon et al., 2016; García et al., 2017; Valenga et al., 2019).

Various work has been done on biodiesel oxidative mechanism which explains that oxidation of biodiesel involves two different pathways but very few focussed upon the effect of antioxidants on the oxidation mechanism. The modelling of reactions to determine the consumption of antioxidant and the organic compound hasn't been taken into popular account. (Rashed et al., 2015; Sazzad et al., 2016; Varatharajan et al., 2017). Hereby, this paper discusses the two different pathways through which oxidation of biodiesel can take place and their modelling for 5 years in MATLAB 2020 in order to understand the amount of degradation that takes place during the oxidation in presence and absence of antioxidant in two different pathways. This will also help to study the oxidation path which gets affected by the presence of antioxidants (Varatharajan et al., 2017).

MATERIAL AND METHODS

The oxidation mechanism of different hydrocarbons has been a subject to various research papers. Any mechanism while processing mainly involves chemical free radical process. Any hydrocarbon contains sites which respond with oxygen by means of the autoxidation component with the old-style revolutionary chain response steps of initiation chain propagation and termination. The initiation step is the development of a free radical that can respond straightforwardly with oxygen to structure a peroxide or hydroperoxide. General mechanism on the basis of which oxidation of unsaturated fatty acid takes place is initiation step. Initially, formation of peroxides takes place, which get additionally corrupted utilizing pathways and the auxiliary items framed utilizing them like acids, aldehydes and polymers. (Saeed et al., 2012).

In the Initiation Period, initiators, for example, heat, light, high energy radiation, metal particle impetuses eliminate a hydrogen ion from the unsaturated fat structure and produce free radicals as appeared in R1. RH speaks to a natural substrate being oxidized (FAME), R* is a carbon-focused alkyl radical got from RH, and HOO* is hydro-peroxyl radical. The carbon-focused radical (R*) shaped in the commencement response are exceptionally receptive, and joins with accessible oxygen, giving peroxy radicals (ROO*) in R2 depicted under Propagation Reaction (Herbinet et al., 2008; Riazi et al., 2017; Riazi et al., 2019; Srinivasan et al., 2019).

The peroxy free radical isn't as receptive as the carbon free radical, yet regardless are adequately responsive and rapidly eliminates another hydrogen by assaulting the frail piece of the FAME structure to shape a hydroperoxide in R3 for example (ROOH*) radical and a R*. The new R* from the above condition would then be able to respond with diatomic oxygen to make the peroxy radicals in R2, and the chain response proliferates. Response of at least two free radicals' prompts bring about end part. This is called Termination Reaction. The essential or chain breaking antioxidants agents responds with this peroxy (ROO*) and hydroperoxide (ROOH*) extremists and intrudes on the proliferation response by arrangement of antioxidant radical for example A*. Essential antioxidants don't restrain the initiation response of the biodiesel degradation (McCormick et al., 2007; Noirbent et al., 2020).

This A* framed, therefore follows two ways, first way prompts the response with antioxidant radical with the diatomic oxygen prompting arrangement of AO2*. This AO2* responds and structure various items. It has been expected by various writings that two pathways would happen alongside rudimentary thought of response. Second way prompts the response between the antioxidant radical with peroxide radical prompting development of ROOA. The estimation of rate consistent has been accepted hypothetically however considering the response component of oxidation of RH (Maia et al., 2011; Focke et al., 2011; Varatharajan et al., 2017).

In this study, two different above discussed pathways has been related to the impact of antioxidant. Modelling of the listed reactions has been done in order to evaluate the effect of antioxidant in the reaction on the whole process.

Using MATLAB 2020, study has been performed in considering the cases in which antioxidant concentration during the reactions has been converted from 0.09 mol/m3 to the final concentration. The concentration of RH (organic substrate) has been taken as 0.03 mol/m3. Hereby, during the modelling, the requirement of rate constants has been raised which have been fulfilled by the previously available studies (Waynick et al., 2005, Zuleta et al., 2012). Table 1 describes the reactions of two pathways along with the assumed kinetic rate constant using different literatures.

Table 1. Reaction Mechanism

Path Number	Reaction Number	Reaction Scheme	Rate Constant Value
1,2	R1	$RH + O_2 \rightarrow R^* + HOO^*$	$1*10^{-4}$ (mol/cm3) s^{-1}
1,2	R2	$R^* + O_2 \rightarrow ROO^*$	$1*10^{10}$ (mol/cm3) s^{-1}
1,2	R3	$ROO^* + RH \rightarrow ROOH + R^*$	$1*10^{-4}$ (mol/cm3) s^{-1}
1,2	R4	$Antioxidant + ROO^* \rightarrow A^* + ROOH$	$1*10^{4}$ (mol/cm3) s^{-1}
1	R5	$R^* + R^* \rightarrow R\text{-}R$	$1*10^{11}$ (mol/cm3) s^{-1}
1	R6	$A^* + O_2 \rightarrow AO2^*$	$1*10^{-2}$ (mol/cm3) s^{-1}
1	R7	$AO_2^* + AO_2^* \rightarrow P$	$1*10^{-2}$ (mol/cm3) s^{-1}
2	R8	$ROOH + A^* \rightarrow ROOA$	$1*10^{-8}$ (mol/cm3) s^{-1}

Figure 1: Path 1 of biodiesel oxidation

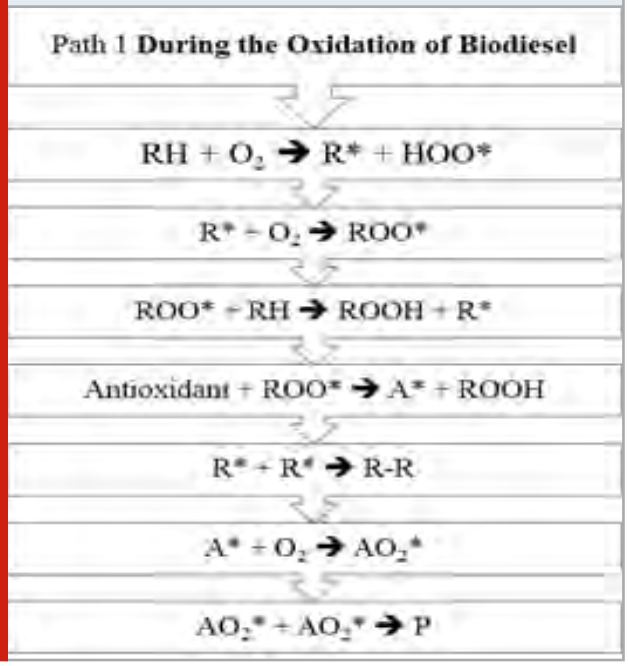
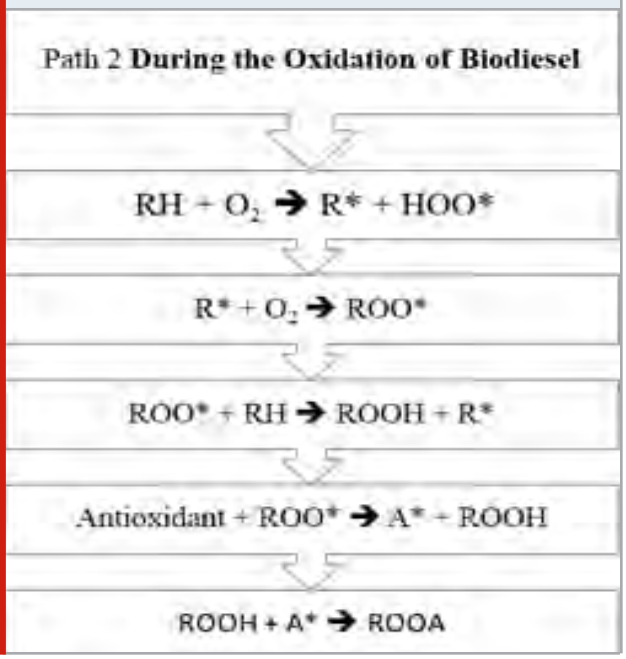


Figure 2: Path 2 of biodiesel oxidation



RESULTS AND DISCUSSION

The modelling part has been done using MATLAB 2020, in which the amount of degradation of organic substrate and antioxidant has been studied that takes place for 5 years, if an oxidation mechanism would happen for both the paths. Determination of the exhausted amount of antioxidant would also take place during the two different pathways in the modelling. As explained above, the RH oxidation decreases in presence of antioxidant. Similar, antioxidant study of safflower biodiesel has also been proved by various literatures which shows the relation of stability of biodiesel with induction period in regards with antioxidants. Other than this, the impact of various antioxidants on refined biodiesel is like the first biodiesel: PY, PG, and TBHQ (tert-butylhydroquinone) gave the best outcome, trailed by BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), DTBHQ, IB, and a-tocopherol showed that antioxidants don't inhibit the mechanism but slow down the process. (Tang et al., 2008; Xin et al., 2008; Karavalakis et al., 2010; Focke et al., 2011; Varatharajan et al., 2017; Devi et-al, 2018).

Similar modelling of reactions of biodiesel oxidation has been done that shows the consumption of biodiesel in the presence and absence of antioxidants in two different pathways for 5 years. Table 2 data obtained from the

modelling in MATLAB 2020 describes the percentage in which the exhaustion of RH takes place in two different cases. It has been seen that, degradation of RH becomes slower in Path 1 in the presence of antioxidant, whereas, not much diference has been seen in Path 2 for presence and absence of antioxidant (Devi et-al, 2018).

Table 2. Degradation of RH for Different Cases w.r.t Time.

Time, Days	Percentage Exhausted in Path 1		Percentage Exhausted in Path 2	
	Presence of antioxidant	Absence of antioxidant	Presence of antioxidant	Absence of antioxidant
200	93.3667	97.0733	98.9933	99.0500
400	95.5700	99.5866	99.9005	99.9088
600	98.0600	99.9483	99.9903	99.9913
800	98.0500	99.9936	99.9990	99.9991
1000	98.6966	99.9992	99.9999	99.9999
1200	99.1366	99.9999	100	100
1400	99.4266	100	100	100
1600	99.6166	100	100	100
1800	99.7460	100	100	100

Figure 3: Degradation of RH in the first path in presence of antioxidant

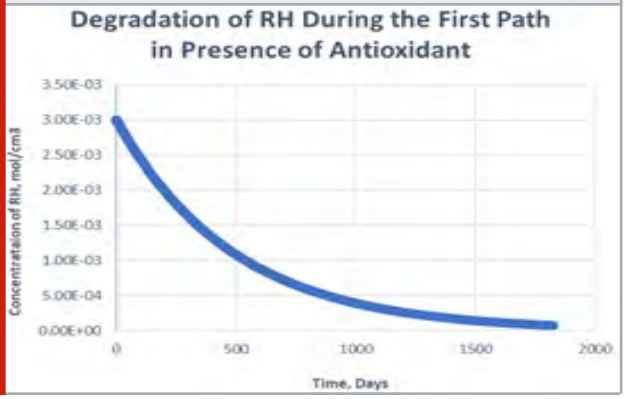


Figure 4: Degradation of RH in the first path in absence of antioxidant.

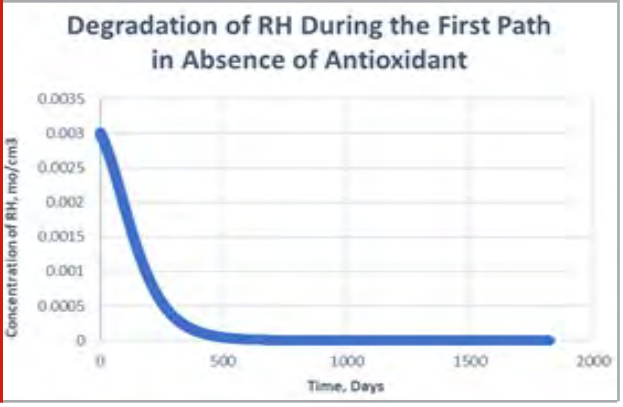


Figure 5: Degradation of RH in the second path in presence of antioxidant

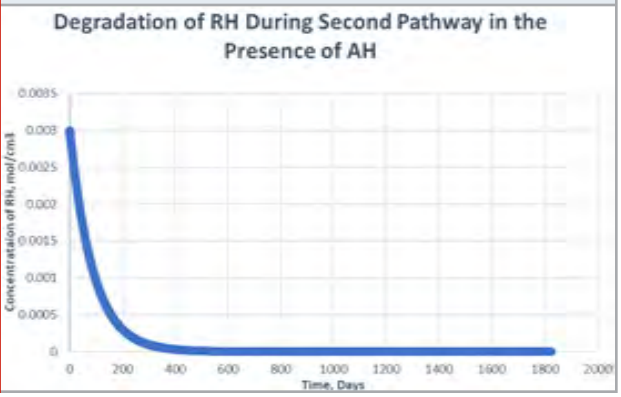
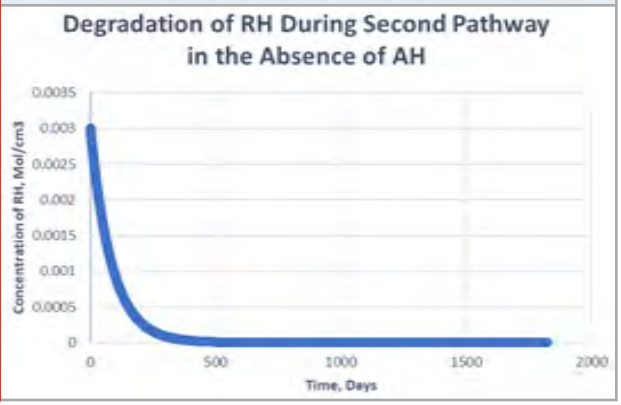


Figure 6: Degradation of RH in the second path in absence of antioxidant.



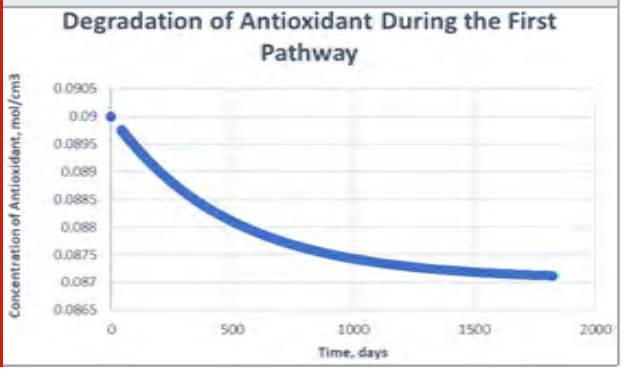
As shown in the plots obtained from modelling in MATLAB, in the Path 1 case, complete degradation of RH takes place in more than 1800 days in presence of antioxidant (Figure 3) and in approximately 1400 days in absence of antioxidant (Figure 4). It clearly shows that antioxidant lowers the rate of degradation of RH. Whereas for Path 2, complete degradation of RH takes place in 1200 days only in exponental plot, although the presence and absence of antioxidant doesn't effects much during the degradation mechanism (Figure 5, Figure 6).

Such study has also been done for soybean oil fatty acid methyl esters (SME) using five different antioxidants. It has been proved that expanding antioxidants stacking (concentration) indicated sharp expansions in action for loadings up to 1000 ppm followed by more modest expansions in action at higher loadings (Varatharajan et al., 2017).

Table 3. Degradation of antioxidant for Two Different Paths w.r.t Time.

Time, Days	Percentage Exhausted	
	Antioxidant in Path 1	Antioxidant in Path 2
200	1.0166	38.9666
400	1.8444	66.2688
600	2.3244	81.4777
800	2.9888	89.9692
1000	2.8577	94.4855
1200	2.9988	97.0800
1400	3.0933	98.4277
1600	3.1544	99.1544
1800	3.1955	99.5544

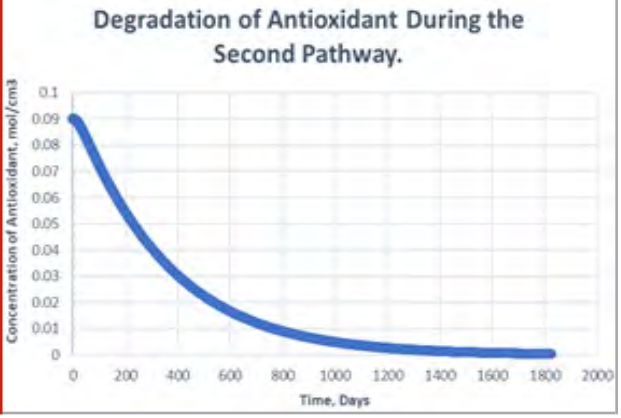
Figure 7: Degradation of antioxidant in the first path



It is clearly observed in table 2 that, antioxidant gets used more in case of second path in order to carry out oxidative stability i.e., it consumed to 99.55% of its initial value. Whereas, only 3.19% of antioxidant gets consumed to carry out the same amount of oxidative stability of RH in case of path one. Hereby, it can be said that if path one is followed, it may decrease the cost of antioxidant to maintain the stability of same amount of RH in exponentially manner (Figure 7, Figure 8). The mechanism followed during the oxidation path of biodiesel becomes dependent of various factors. Since, addition of antioxidants causes storage stability and about association between the physical and compound attributes of biodiesel with natural variables, for example, light, metal pollution, shading changes, and dregs development. The difference between the consumption of antioxidant in different paths arises, due to formation of products like aldehyde, ketones etc, if secondary

Other than this, various other researches involve execution of antioxidants assessed for treating biodiesel got from soybean oil, rapeseed (low-erucic) oil, utilized cooking oil, fat (meat), and palm oil feedstocks proved that different antioxidants provide different change in concentration of same origin of biodiesel. However, no such work came across which focusses upon the routes of oxidation and effect of antioxidants on different routes. However, the present study becomes relevant as comparable to previous researches since the common antioxidant and common biodiesel concentration change takes place during the modelling (Dunn, 2005; Dunn, 2008; Ashok et al., 2017; Varatharajan et al., 2017). During the modelling part, in the presence of antioxidant, since the antioxidant gets reacted during the reaction and product formation takes place that shows the consumption of antioxidants as well. Table 3, Figure: 7 and Figure 8, data obtained from simulation part describes the percentage in which the exhaustion of antioxidant takes place in the two different cases (Varatharajan et al., 2017).

Figure 8: Degradation of antioxidant in the second path.



mechanism is followed (Kivevele et al., 2011; Rashed et al., 2015).

CONCLUSION

Degradation of aromatic compounds is the common phenomenon observed in industries. Such degradation also takes place for hydrocarbon compounds like biodiesel. The objective of the paper is to study about the consumption of antioxidant at different paths of biodiesel oxidation along with the degradation of biodiesel (RH) during the two paths. Both the studies have taken place keeping the duration of 5 years. It has been concluded that antioxidant has affected the path one process more as compared to the path two process during the degradation mechanism of biodiesel in 5 years. Whereas consumption of antioxidant in path 1 is only approximate 3% as compared to 99% in path two. Therefore,

oxidative degradation carried from path one can be controlled with lower amount of antioxidant whereas no such effect of antioxidant takes place during reactions of

path two even after its complete consumption. This data, can become useful for the industries which stores any organic substrate (like biodiesel), since the storage of such compounds can become harmful specially when chance of degradation like oxidation, corrosion etc are common for few compounds. To obtain concentration vs. time plot for biodiesel and antioxidants, the concentrations may be measured by techniques like Spectrophotometry or HPLC. Such type of mathematical models together with enthalpy balance, can be developed to predict the potential hazardous oxidation leaks in storage tanks. Such studies would help in developing safer practices.

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Inhibition Kinetics of Immobilized Cathepsin B on the Surface of Gold Nanoparticles

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ABSTRACT

Present study is based on the concept of green synthesis of gold nanoparticles using cathepsin B and functionalization of cathepsin B on their surface which are involved in cancer. Classical citrate method was used to reduce chloroauric acid with tri-sodium citrate in an aqueous solution to produce citrate gold nanoparticles (C-GNPs). Further, these nanoparticles were immobilized with cathepsin B in order to study the conformation changes during and after function alization. The C-GNPs and cathepsin B immobilized gold nanoparticles (Cat-B-GNPs) were characterized by UV-VIS spectroscopy, TEM, DLS, Zeta potential, SEM and FTIR to confirm the size, functional group interactions, production, stability, crystalline nature, size and shape distribution. However, FTIR studies confirmed the attachment of cathepsin B in the gold nanoparticles. The variation in 2D and 3D conformation of cathepsin B during immobilization was studied by Circular Dichroism and Fluorescence spectroscopy, respectively. It was observed that total changes in α- helix, stand, and turn is 45.41%, 34.75%, 76%, respectively. After immobilization, a 54.91% changes in tertiary structure was found. The change in 2D & 3D conformation of cathepsin B after immobilization may improve cathepsin B potency which would be verified by enzyme kinetics inhibition. Results showed that E-64 significantly inhibited Cat-B-GNPs activity with an IC_{50} of 4.88 µM and inhibition was a non-competitive type of inhibition with a K_i of 5.09 µM. Michaelis-Menten constant K_m of cathepsins B and Cat-B-GNPs were determined by using Z-Phe-Arg-AMC as substrates and E-64 as a model inhibitor.

KEY WORDS: CATHEPSIN B, CIRCULAR DICHROISM, E-64, GOLD NANOPARTICLES, Z-PHE-ARG-AMC.

INTRODUCTION

In malignant tumors, the expression of cathepsin B is highly upregulated and secreted into the extracellular environment (Jakoš et al., 2019) that are required for cancer progression (Poreba et al., 2019) Overexpression of cathepsin B has been demonstrated in various types of cancers, including breast cancer, glioma, melanoma, and oesophageal, pancreatic, colon, renal and prostate cancer. These endogenous tumour specific characteristics provide valuable tools for the design of formulations that will only be activated in the tumour microenvironment (Mijanovic, et al., 2019). Cathepsins act on the drug release process from the nanomaterials. Drug release behaviour studies with nanoparticles showed that the nanoparticles had the greatest release of contents in the presence of cathepsin B at lysosomal pH, indicating the requirement of cathepsin B cleavage for sufficient drug delivery.

There have been various nanoparticles based delivery strategies have been developed such as rotaxane modified mesoporous silica nanoparticles to improve targeting of anticancer drug doxorubicin (Anderson et al., 2017), PEGylated PLGA nanoparticles encapsulated paclitaxel

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and etoposide used in cancer (Wang et al., 2015), camptothecin containing nanoparticle drug conjugated with bevacizumab against renal cell carcinoma (Voss et al., 2017), methoxy-poly (ethylene glycol) aldehyde conjugated with doxorubicin and Curcumin against HepG-2 cancer cells (Zhang et al., 2016) and doxorubicin carbonane conjugated polymeric nanoparticles used for cancer therapy (Hejian et al., 2015).

Dendrimer methoxy polyethylene glycol which conjugated with doxorubicin using cathepsin B responsive drug to target cancer cells (Ahn et al., 2009). M.K shim et al developed carrier free nanoparticles of cathepsin B cleavable peptide Phe-Arg-Arg-Gly conjugated with doxorubicin to target cathepsin B (Shim et al., 2019). The concept of using nanoparticles as a carrier for anticancer agents is based on the observation that drugs appear to be inactive while bound onto nanoparticle surface and they elicit their cytotoxic action once the nanoparticles digested within the tumour microenvironment (Li et al., 2000; Rudzicska et al., 2020). Nanoparticle delivery of specific cathepsin inhibitors can enable targeted recognition of cancer cells and effective inhibition of intracellular activity of cathepsins.

PEGylated liposomes with an epoxide based cathepsin B inhibitor NS-629 conjugated through a lipid linker (LNC-NS-629) used in Cathepsin B targeted delivery of therapeutics or diagnosis to the cancer site (Farooq et al., 2018). Poly-lacto-co-glycolic acid (PLGA) nanoparticles and chitosan nanoparticles conjugated with cystatin were successfully developed using an optimized method that retains the inhibitory activity of the encapsulated cystatin. Similarly, PEGylated liposomes conjugated with irreversible epoxyketone cathepsin inhibitors Carfilzomib and bortezomib were successfully developed to target cancer (Stern et al., 2008; Deng et al., 2020).

Cathepsins inhibitor have been shown to be effective anticancer therapeutic agents and delivery with nanoparticle systems can substantially improve their bioavailability, increase circulation time and achieve specific accumulation in tumor tissues. Enzyme inhibition has always been an important field of study, not only because of its usefulness in providing valuable information on fundamental aspects of enzymatic catalysis and metabolic pathways, but also for its implications in pharmacology and toxicology. Compared to reversible inhibition, the kinetics of irreversible inhibition has received relatively little attention. However, it is well established that irreversible modification of enzyme activity is important for studies on the nature of functional groups essential to enzymatic catalysis; such studies cannot be conducted with reversible inhibitor.

In the study presented here, we synthesized citrate mediated gold nanoparticles which functionalized with cathepsin B. The main goal of the study is to detect the unfolding and folding pathway of cathepsin B during the synthesis of Cat-B immobilized GNPs (Cat-B-GNPs). The characterization of C-GNPs and Cat-B-GNPs was done by UV-VIS spectroscopy, TEM, DLS, Zeta potential, SEM and FTIR. Furthermore, the enzyme inhibition kinetics was done to evaluate the efficacy of free enzyme against immobilized enzyme (Cat-B-GNPs) using Z-Phe-Arg-AMC substrate and E-64 as a model inhibitor.

MATERIAL AND METHODS

Tetrachloroauric [III] acid [$HAuCl_4$] was procured from sigma Aldrich Co. USA. Cathepsin B from human liver, the irreversible inhibitor E-64 [L-3-carboxy-trans-2,3-epoxypropionyl- leucylamido-(4-guanidino) butane] and the fluorogenic amido methyl coumaryl substrate Z-Phe-Arg-AMC were purchased from Merck. DTT (dithiothreitol), tri-sodium citrate andEDTA were purchased from HiMediaLaboratories, Mumbai, India. All other reagents were of highest analytical grade available. Active forms of cathepsin B were stored at 4°C in 50 mM sodium acetate buffer (pH 5.0) containing 10 mM methyl methane-thiosulfonate.

In vitro synthesis of citrate encapsulated gold nanoparticles (C-GNPs): In vitro production of C-GNPs was done by reduction of tetra chloroauric [III] acid $HAuCl_4$ with tri-sodium citrate (NaCt) following Turkevich method (Turkevichet al., 1951). Citrate mediated synthesis of GNPs was performed by incubating1mM HAuCl4 with 1% tri-sodium citrate solution in 5ml double distilled water at 100 on a hot plate with continuous stirring at 350 rpm until homogenous solution achieved. C-GNPs gradually produce as the NaCt reduces the gold ion (Au^{3+}) into the gold atom (Au^0). The confirmation of synthesis of C-GNPs was authenticated with change in color of reaction from transparent to light yellow, to grey, purple and then eventually in deep ruby red for gold nanoparticles. Confirmation of synthesis of C-GNPs was confirmed by the UV absorption spectroscopy, TEM, DLS, Zeta potential and SEM. After the completion of the reaction, the reaction mixture was removed from hot plate and air cooled at RT.

Immobilization of cathepsin B over the surface of C-GNPs: Immobilization of cathepsin B over the surface of C-GNPs was done by incubating 33µg/ml with 1ml C-GNPs for 2 hours at room temperature and subsequently, 0.05M of NaCl to the reaction mixture and incubating for 1hour, to increase ion concentration in the solution. CathepsinB immobilized on the surface of GNPs during the transfer of citrate to the NaCl, which prevents the aggregation of GNPs and ensure stability. After the reaction completion, 50% v/v of ethanol treatment was used to remove unbound cathepsin B, and further by centrifugation (10 min) nanoparticles were collected and used further for characterization (Iram et al., 2019).

Characterization of in vitro synthesized C-GNPs and cathepsin B capped gold nanoparticles (Cat-B-GNPs): C-GNPs and Cat-B-GNPs were characterized by various techniques, UV–vis spectrophotometer measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC), operated at a resolution of 1 nm. Transmission electron microscopy (TEM) was done on TecnaiTMG2Spirit BioTWIN FEI Company operated at an accelerating voltage of 80 kV. The sample was prepared by drying a drop of Cat-B-GNPs solution on carbon coated TEM copper grids followed by measurements on TEM.Scanning electron microscopy (SEM) analysis of synthesized Cat-B-GNPs was operated by the use of a Sigma, Zeiss HR-SEM machine. The mean particle size of Cat-B-GNPs was measured with a dynamic light scattering (DLS) particle size analyzer (Zeta Sizer Nano-ZS, Model ZEN3600, Malvern Instrument Ltd).

The particles were sonicated by using Sonic & Material Inc., at 30 W for 1 min. Mean particle size was the average of triplicate measurements for a single sample. The surface charge of C-GNPs and Cat-B-GNPs were measured using a Zeta Sizer Nano-ZS, Model ZEN3600. The confirmation of binding and secondary structure of cathepsin B at the surface of GNPs was done by Fourier transform infrared spectroscopy (FTIR). FTIR spectra of the sample were recorded on a Shimadzu FTIR-8201 PC instrument operated in the diffuse reflectance mode at a resolution of 4 cm^{-1}. To obtain good signal-to-noise ratios, were taken in the range 400–4000 cm^{-1}.

Circular dichroism (CD) Studies: Conformational changes in the secondary structure of cathepsin B due to immobilization over the surface of C-GNPs were performed on a Jasco J 800spectropolarimeter using a 1 mm path length cell. The machine was aligned with D-10-camphorsulfonic acid. The nitrogen flow was fixed at 10 liters/min. The CD spectrum was monitored over a variable wavelength scan (200-250nm). The CD in millidegree obtained over the wavelength range of 190 to 250 nm was converted to mean residue ellipticity (MRE,) using the following conversion: MRE = CD (in mdeg)/cpnl, where cp is molar concentration of protein, n is number of amino acid residues in the protein,and l is path length (in mm).Native and Cat-B-GNPs samples were put in 1 mm path length cuvette and spectra were captured in the variable wavelength scan (200 to 250 nm). Every spectrum was the mean of 3 scans and was recorded at an interval of 1 nm wavelength. The enzyme concentration in these tests was 33µg/ml.

Study of conformational changes in cathepsin B by spectrofluoremeter: 3D Conformational changes in the cathepsin B during immobilization over the surface of C-GNPs were performed on Agilent Carry Eclipse Fluorescence Spectrophotometer–MY16040008 using the FLR software provided by the manufacturer. The fluorescence emission was recognized in the range of wavelength 300-400nm after exciting the protein at 280nm. Both the excitation and emission silt width were set at 5nm & voltage is 600 V. The excitation & emission spectra were smoothed with Savitzky-Golay smoothing factor 10. Percent changes in fluorescence intensity (FI) of cathepsin B during immobilization was calculated from the following equation: % changes in FI = [(FI of native cathepsin B – FI of cathepsin B with GNPs at different time)/FI of native cathepsin B] × 100.

Enzymatic activity of Cat-B-GNPs against Fluorogenic methyl coumaryl substrate (Z–Phe–Arg–AMC): Standard conditions used to determine the hydrolytic activity of cathepsin B (at pH 6.5), against the fluorogenic substrate Z–Phe-Arg–AMC (stock solutions prepared in dimethyl sulfoxide) at 37°C according to the method of Barrett and Kirschke (Barrettet al., 1988). After 30 min of incubation with substrates, the hydrolytic reaction by this proteinase was terminated by adding 5% of TCA (trichloro acetic acid) containing 2.5 ml HCl. The intensity of fluorescence of the mixture was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. One unit of enzyme activity was expressed as the amount of enzyme that can hydrolyze methyl coumaryl amide substrates and release1 µmol of amino methyl coumarin within 1 min of reaction at 37°C.

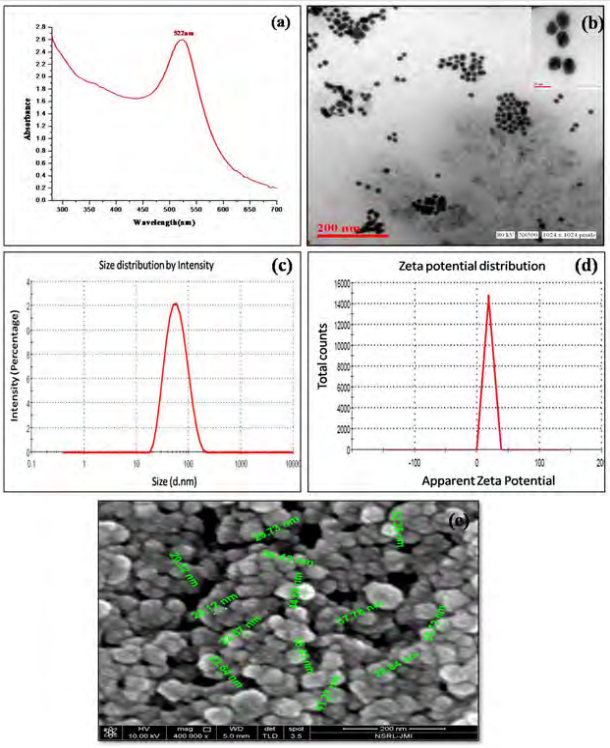
For the Lineweaver–Burk analysis, Cat-B-GNPs (250ng/ml) was incubated with inhibitor at 12µM and 24µM assayed at increasing concentrations of substrate (10µM, 20µM, 30µM,40 µM) in 50 mM sodium potassium phosphate buffer (pH 6.5) containing 2 mM EDTA and 4mM DTT was separately added. The fluorescent intensity of free amino methyl coumarin release from the hydrolysis of methyl coumaryl amide substrate due to this proteinase was measured continuously by a fluorescence spectroscopy (excitation, 350 nm; emission, 420 nm; slit, 5 nm) at 37°C. The initial velocities (v) were calculated from the reaction curve, i.e. the slop of the initial linear cure. Lineweaver-Burk doublereciprocalplot of substrate concentration (s) versus initialvelocity (v) was used to calculate the Michaelis constant (K_m) and maximum velocity (V_m).

RESULTS AND DISCUSSION

Preparation of C-GNPs and Characterization: A typical citrate reduction method of gold nanoparticles at 100 takes 30-40 minutes for the characteristic ruby red color to appear in the solution. Three different aspects are of crucial importance for obtaining desirable size of nanoparticles (i) the temperature, (ii) the pH, and (iii) concentration of Au atoms (Ojea-Jiménez et al., 2009). The synthesis of C-GNPs was confirmed by a gradual transform in color starting from light grey to

characteristic ruby red color subsequent by incubation of citrate and Au atom. The transformation in color is due to the surface plasmon resonance of C-GNPs. Further, it was confirmed by UV-Visible spectroscopy, the absorption band of C-GNPs showed an intense peak at 522nm (Fig. 1a) (Sakellari, et al 2020).

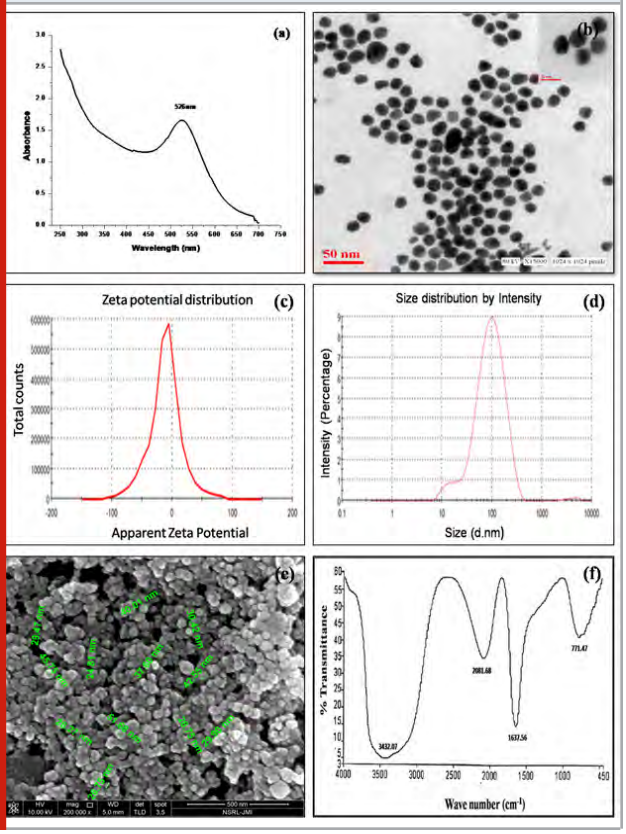
Figure 1: Characterization of C-GNPs under (a) UV–vis spectroscopy (b) Transmission electron microscopy (c) Dynamic light scattering (d) Zeta potential and (e) Scanning electron microscopy



High resolution images of C-GNPs were obtained using TEM and most particles were estimated to be monodispersed and spherical at an average size of approximately ~18 nm (Fig.1b). In addition, DLS was carried out to estimate the hydrodynamic radius of C-GNPs was obtained to be 49.53 nm (Fig.1c). C-GNPs with a negative charge were found to be stable and their zeta potential values were found to be -1mV (Fig. 1d). SEM image clearly shows the spherical and uniformly distributed C-GNPs (Fig.1e).

Immobilization of cathepsin B on C-GNPs and their characterization: Electrostatic repulsion between negatively charged cathepsin B and negatively charged spherical C-GNPs should hinder the protein adsorption. However, in the present study strong interaction was observed. The strong binding of cathepsin B to nanoparticle surface occurred most probably due to the NaCl, which start dislocation of citrate ions towards the Na ion. Cathepsin B, a strong stabilizing agent with Cys, Asp and His in the active site which makes nanoparticle more stable and prevent aggregation. Immobilization process of cathepsin B on the surface of GNPs is spontaneous process where most of type interactions involve such as hydrophobic interaction, and ionic interaction. Hydrophobic interactions are due to attraction between hydrophobic parts of the cathepsin B and the gold surface resulting, the formation of non-covalent bond. Ionic interaction are formed between positively charged amino acid and negatively charged gold nanoparticle surface.

Figure 2: Characterization of Cat-B-GNPs under (a) UV–vis spectra (b) Transmission electron microscopy (c) Zeta potential (d) DLS (e) Scanning electron microscopy and (f) FTIR



Cathepsin B stabilized/immobilized GNPs (Cat-B-GNPs) have shown a decrease in intensity of the peak with a noticeable red shift from 522 nm to 526 nm in comparison to C-GNPs (Fig. 2a). The red shift in SPR confirms a change in the surface chemistry of C-GNPs due to immobilization of cathepsin B on their surface. Generally, GNPs single absorption peak in the visible range between 512-550 nm, because of surface Plasmon resonance display and show heavy absorption of visible light at 520 nm. TEM is another important aspect for characterization shows the high resolution TEM image of Cat-B-GNPs. The size of Cat-B-GNPs has been determined by measuring the diameter of whole particles on TEM images.

TEM calculates the size of nanoparticles by directly transmitted electrons which give the information about only inorganic core and doesn't include hydration layer. High resolution images of Cat-B-GNPs were obtained using TEM and most particles were estimated to be monodispersed, spherical and evenly dispersed at an average size of approximately ~20 nm (Fig. 2b). Their shapes were found to be spherical and monodispersion state, this is because of negatively charged layer of cathepsin B, which repel from each other. The cathepsin B molecules adsorb onto the surface of the C-GNPs and make a layer of negatively charged ions. The resulting electrostatic interaction provides an interparticle force strong enough for the particles to stay separated.

The concentration, distribution, ionization, exposure of charged moieties, adsorption of the particles can be recorded by zeta potential. The change in zeta potential of Cat-B-GNPs was observed and it was found to be –11.4 mV (Fig. 2c). Further, DLS was conducted to estimate the hydrodynamic radius of Cat-B-GNP was found to be 77.29 nm (Fig. 2d). SEM results revealed that the particles are smaller in size and uniformly distributed. However, the particle size of the Cat-B-GNPs was estimated to be in the range of 25-35nm (Fig. 2e).

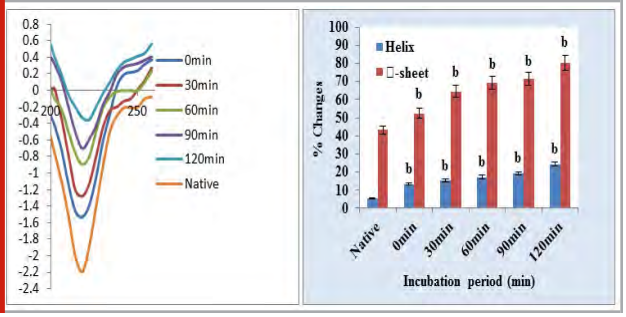
In order to detect involvement of different functional groups of protein to functionalize GNPs, FTIR was carried out (Fig. 2f). There are two regions 1700–1600 cm^{-1} 1550–1500 cm-1 in the spectrum, which are unique to the protein secondary structure, called as amide I and II bands. These provide valuable information on conformational changes of the protein when immobilized with the nanoparticles. The amide I band (C=O stretch) has a correlation with the secondary structure of protein. The immobilization of cathepsin B over C-GNPs was confirmed by observing a characteristic peak at 1637.56cm-1corresponding to the presence of amide groups.

Further smaller peak observed at 771.47cm-1 correspond to C-N of aliphatic amines stretch of peptide bond also confirms the immobilization. In this study a novel method has been developed to immobilize cathepsin B over gold nanoparticles surface. During immobilization over the surface of gold nanoparticles cathepsin B undergo several changes in their secondary and tertiary structure and then finally adopts its native structure with less variations which do not alter cathepsin B property. Only some reports are presented where the folding & unfolding of protein have been studied using techniques such as circular dichroism (CD) spectroscopy, and fluorescence spectroscopy.

In this study, citrate mediated gold nanoparticles have been stabilized/immobilized by cathepsin B. The CD spectra of cathepsin B show a clear change in the protein structure during immobilization over the surface of GNPs in the far UV region between 200 and 240 nm. A more detailed analysis of the spectrum using K_2D_3 software which allow estimating the percentage of secondary structure in each CD spectrum. The α helical structure of the protein in the far UV region is characterized by negative peaks at 208-210 and 222 nm and positive peaks between 190 and 192 nm. The results were expressed as MRE (mean residue ellipticity) in deg•cm2•dmol^{-1}, which is defined as: MRE = θ obs/ (10Cpnl), Where θ obs is the CD in milli degrees, n is the number of amino acid residues, l is the path length of the cell, and Cp is the mole fraction. Helical content of cathepsin B was calculated from the MRE values at 222 nm using the following equation: % α helix = (MRE222 – 2340/30300) × 100.

The structure of cathepsin B is comprised of a helix (14%), β sheets and turns with 35% residual part in various structures (Garnier et al., 1978). The molecular mass of cathepsin B is 38 kDa with three disulfide bridges and a solitary free cysteine amino acid among cumulative 339 amino acids demonstrates the Native and cathepsin B-Gold in far-UV CD spectra (Cavallo-Medved et al., 2011). There was an almost 45.41% in α-helical structure and 34.75% changes observed in -sheet in the secondary structure after immobilization of cathepsin B (Fig. 3).

Figure 3: (a) Far UV-CD spectra of Cathepsin B and Cat-B-GNPs at different incubation period (b) Bar graphs are representing % α-helix & β-sheet content of cathepsin B during immobilization on the surface of GNPs. % α-helix & β-sheet content was interpreted through K2D2 software (http://cbdm-01.zdv.unimainz.de/~andrade/k2d2/). Values (Percentage) are the average of three determinations. Significantly different from native cathepsin B at a p<0.01



This can be expected to conformational change from the native state of protein after binding to the nanoparticle surface. α helical structure has two negative bands in 210 and 222 nm. With the progress of the reaction (till 2Hrs) ellipticity gradually decreases with time due to the extent of α-helical structure of cathepsin B structure is reduced as a result of immobilization with GNPs. The above information clearly showed that there was a change in 2D conformation of cathepsin B after immobilization over GNPs but 3D conformation didn't alter significantly.

This implies that slight loss in secondary structure does not lead to tertiary structure loss cm^{-1}.

Fluorescence studies: In the beginning of the reaction, very strong fluorescence signal was observed because at the beginning gold was present in the form of Au^{+0} ions and it interacts with cathepsin B through hydrophobic interactions. As the incubation period proceeds (at 30 min), less fluorescence signal was obtained because the exposure of aromatic amino acid gradually decreases which enhance hydrophobic interactions and cause folding of cathepsin B. With the progress of the reaction (till 120min) fluorescence intensity decreases with time due to involvement of hydrophobic sites of cathepsin B in the interaction with GNPs. Ultimately, after completion of the reaction, cathepsin B adopts its native structure (Fig. 4).

Figure 4: (a) Changes in protein fluorescence intensity (fluorescence excitation 280nm) in the Cathepsin B during immobilization at different incubation time (0min, 30min, 60min, 90min, and 120 min). (b) Significantly different from native cathepsin B at a p^a<0.01.Significantly different from native cathepsin B at a pb<0.001

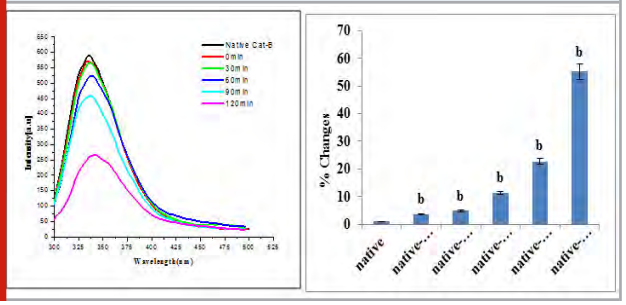
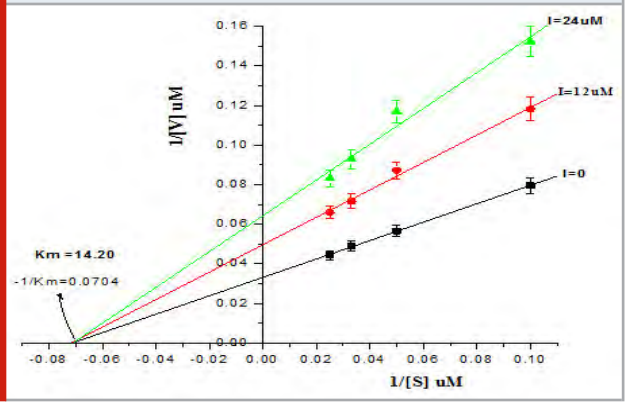
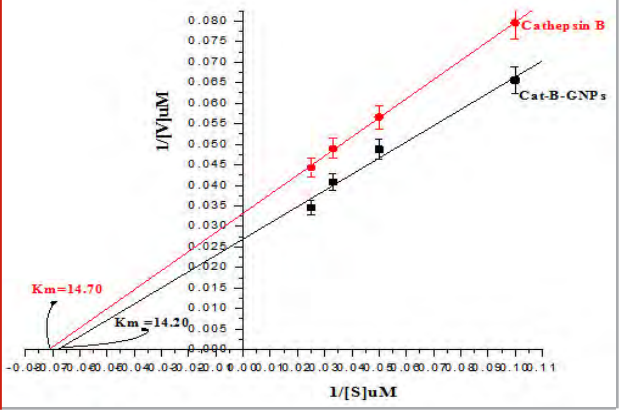


Figure 5: Line weaver–Burk plot of Cat-B-GNPs inhibition by different concentration of E-64 at 37.



Inhibition Kinetics Studies: The inhibitor kinetics studies were attempted to explain how an inhibitor acts on enzyme and predict its efficacy. The kinetic constants K_m and K_i are critical to understand enzymatic action on controlling metabolism of an organism. We studied enzyme inhibition kinetics of E-64 on Cat-B-GNPs. The Michaelis-Menten constant K_m was determined by Lineweaver-Burk plot in which the reciprocals of substrate hydrolysis (1/V) for inhibitor concentration were plotted against the reciprocals of the substrate concentrations by fitting the resulting data in ORIGIN 6.1 (Fig.5). The K_m value for the both cathepsin B and Cat-B-GNPs with Z-Phe-Arg-AMC substrate was found to be 14.70 µM and 14.20 µM at 37□ (Fig. 6).

Figure 6: Line weaver–Burk plots of both cathepsin B and Cat-B-GNPs to calculate K_m against Z-Phe-Arg-AMC at 37□



The Cat-B-GNPs activity was measured at different concentrations of substrate Z-Phe-Arg-AMC (10, 20, 30, and 40 µM). The K_m value for the Cat-B-GNPs with Z-Phe-Arg-AMC was found to be 14.20 µM at 37□ (Fig. 5). The reciprocals of substrate hydrolysis (1/v) were plotted against the different enzyme concentrations (Fig.7). The inhibition constant K_i was determined by Dixon method, hydrolytic activity of Cat-B-GNPs were measured in the presence of 10µM and 30µM Z-Phe-Arg-AMC, at increasing concentrations of inhibitor at 37□ for 30 min (Fig. 6). The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and the K_i was determined by fitting the data using ORIGIN 6.1.The double reciprocal Lineweaver Burk Plot demonstrated the non-competitive mode of inhibition of Cat-B-GNPs by E-64, obtained values of Ki from Dixon plot was found to be 5.09 µM (Fig. 8). The E-64 inhibitor was found to inhibit Cat-B-GNPs with an IC_{50} value (50% inhibitory concentration) of 4.88 µM (Fig. 9). The inhibition of Cat-B-GNPs followed a linear pattern with increasing concentrations of the inhibitor.

In the present study, E-64 displayed a significant concentration dependent inhibition of Cat-B-GNPs using Z-Phe-Arg-AMC as a substrate. To elucidate the mechanism of Cat-B-GNPs inhibition by E-64, kinetic studies of enzyme activity were performed. The relationship between substrate concentration and reaction velocity was in good agreement with Michaelis–Menten equation. Hence, the result demonstrated that mechanism

of Cat-B-GNPs inhibition was of non-competitive nature. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicate, and the average value was considered throughout this work.

Figure 7: Linear fit between velocity [v] and enzyme concentration.

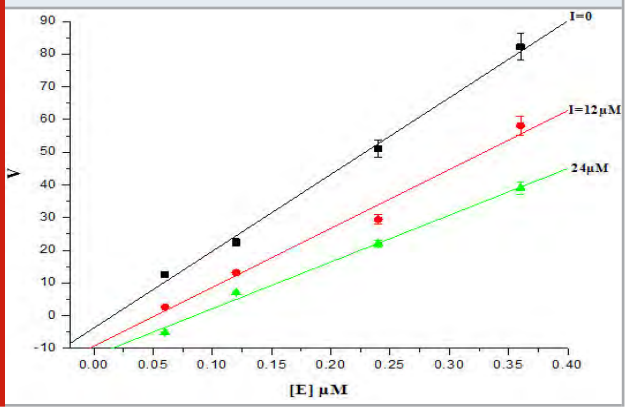


Figure 8: Dixon's Plot: Enzymatic activity of the Cat-B-GNPs was estimated using the substrate Z-Phe-Arg-AMC (10µM and 40µM) at different concentrations of E-64. Reciprocals of the reaction velocity were plotted versus the E-64 concentration. The straight lines indicated the best fit of the data obtained. The inhibition constant K_i was calculated from the intersection point of the two plots.

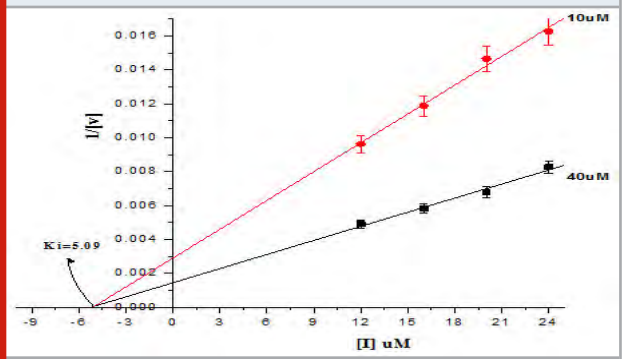
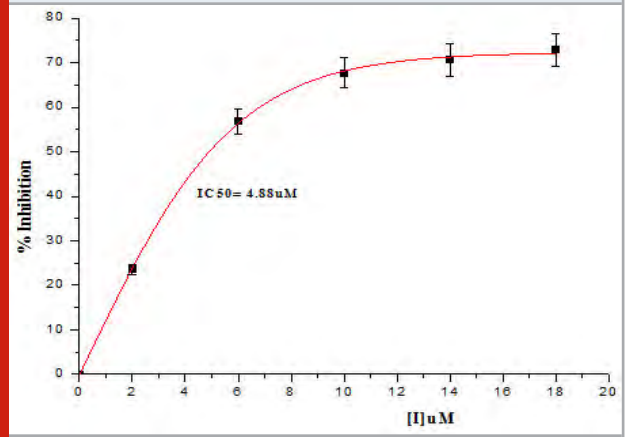


Figure 9: Cat-B GNPs inhibition curve for E-64.



CONCLUSION

The Present study reveals that sodium citrate behaves as a reducing and cathepsin B as a capping agent. After controlled synthesis their physic-chemical characterization is important. It is suggested that for the toxicological studies nanomaterials must have to confirm the crucial set of characteristic which includes size, shape, surface, charge, condition of scattering, chemical composition, surface range and surface chemistry. Surface chemistry of nanomaterial is often another essential step which includes modification of the surface of nanoparticles by using functionalizing/ immobilization. During the process of immobilization, cathepsin B undergoes several stages of unfolding to folding and finally, after complete immobilization on C-GNPs, adopts a final configuration with variations in 3D & 2D structures. Further, we investigated the kinetic mechanism of Cat-B-GNPs and the interaction mechanisms with E-64 inhibitor.

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Statistical *In vitro* Model for Upscaling Biofilm of *Chroococcidiopsis cubana* by Media Optimization and its Protocol for DNA Extraction

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ABSTRACT

Manual process for the optimization of different salts for growth of bacteria is labour intensive work and has low precision. In this study, we had used design expert software for biofilm yield optimization of a strain of *Chroococcidiopsis cubana*. *C. cubana* was exposed on outer surface of the monuments due to adverse environmental conditions and formed the blackish biofilm on it. This biofilm was grown in culture media and their DNA was extracted for strain confirmation. Strain was confirmed by 16s rRNA gene sequence using sanger sequencing. The response surface method was used to optimize the concentration of two main components $NaNO_3$ and K_2HPO_4 among various salts of BG 11 media. RSM was studied by ANOVA coefficient estimation using F – test with very low probability value. The obtained goodness of fit was significant (R^2 = 0.99). Estimation of coefficient was used for calculation of t and p – values and decided their significancy. The model having p value less than 0.05 was considered for optimization. Desirability of optimization was resolved from contour plot having concentration of $NaNO_3$ = 13 and K_2HPO_4 = 6.50 for optimum biofilm yield. Using these parameters, the *in vitro* model of *Chroococcidiopsis cubana* resulted in a yield of 20 g/l biofilm in 10 days.

KEY WORDS: ANOVA, ENDOLITHIC BIOFILM, RESPONSE SURFACE METHOD, 16S RRNA GENE SEQUENCING.

INTRODUCTION

Biofilm is defined as sessile microorganisms growing on solid surface and embedded in matrix of extracellular polymeric substances (Garrett et al., 2008). These microorganisms include several bacteria like *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* etc as well as some blue green algae like *Nostoc* sp., *Phormidium* sp., *Microspora* sp., *Chroococcidiopsis* sp. etc (Hancock 2013; Ljaljevic-Grbic et al., 2010; Miller et al., 2009). *Chroococcidiopsis* sp., a blue-green alga, has desiccation tolerance due to the thick polysaccharide sheath on the outer surface of the cells (Knowles and Castenholz, 2008). In some species such sheaths have the ability to self-recognise the surfaces. Due to this, cells auto-aggregate mostly at the bottom of the broth culture flask. Hence, exopolysaccharides are also known as auto-agglutinins. This is the first stage in the whole process of biofilm formation (Trunk et al., 2018).

The study of biofilm formation has importance in a broad scale of industrial application such as health and cosmetics products, food, water, paper mills, medical health and pharmaceutical (Molobela and Ilunga,

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2012). *In vitro* production of biofilm is a laborious and cumbersome task. Hence, it is necessary to optimise some components for enhanced biofilm yield through a reliable and successfully working statistical tool like response surface methodology (RSM) (Bratchell, 1989; Lundstedt et al, 1998; Mirhosseini and Tan, 2009; Khuri and Mukhopadhyay, 2010). Growth rates and yield of the species are dependent on the concentration level of $NaNO_3$ and K_2HPO_4 (Rippka et al., 1988).

In the present work, *Chroococcidiopsis* sp. was isolated from biofilm and obtained pure culture by streak plate method. Central composite design (CCD) of RSM has been applied to obtain the optimized concentration of two main salts components $NaNO_3$ and K_2HPO_4 of BG 11 media for in vitro biofilm yield. DNA was extracted from biofilm and was amplified using 16s rRNA primer for strain confirmation.

MATERIAL AND METHODS

Isolation of Microorganism: Surface biofilm of the endolithic *Chroococcidiopsis* sp. was collected during post monsoon period from the dome in the main building of Faculty of Arts, The M.S. University of Baroda, Vadodara, India. The biofilm was immersed in petri plate having sterile distilled water and kept for 24h. Clump of biofilm was segregated with the help of needle and isolated by streak plate method in agar (Rippka et al., 1988). Culture was maintained on agar plate and sub cultured every two weeks.

Growth medium: BG 11 medium as described by Rippka et al., (1988) having pH 7.2 was prepared as control. A 250 ml Erlenmeyer flask containing 100 ml of media was inoculated and incubated at 27 – 30 ^{0}C temperature in 2500 Lux white light for 14h and 2h rotatory shaker at 100 RPM for 10 days. The biofilm was separated by centrifuging at 4000 RPM for 10 mins. Supernatant was discarded and yield of biofilm was measured. The same procedure was repeated for all the experimental flasks.

Experimental design for optimization: CCD was selected to optimize the concentration of $NaNO_3$ and K_2HPO_4 for enhancing the yield of *Chroococcidiopsis* sp. The experiment was designed by the Design Expert 7.0.2 (stat-ease, USA). The range of the variables of $NaNO_3$ and K_2HPO_4 were selected from preliminary study. The lowest and highest values of the variables have mentioned in table 1. Central composite design with 22 factorial gives 4 star points and axial points having 5 replicate at the centre as central point leading to 13 runs were engaged for the optimization of the concentration (Bradley, 2007). For statistical calculation, the variables were coded by equation 1:

$$x_i = {}^{x_1 - x_0}/_{\Delta x} \quad (i = 1, 2, .. q) \qquad (1)$$

x_i is the dimensionless value of variable 1, x_1 is real value of variable 1, x0 is centre point value of x_1 and x is step change.

CCD is second order level design. In this design, the dependent variable gives information about interaction between factors in their relation to the dependent variable. 2nd order polynomial equation 2:

$$\hat{Y}_i = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i,j=1}^{2} \beta_{ij} x_i x_j \qquad (2)$$

$\hat{Y}_i$ denoted predicted response, xi and xj input variables, β_0 intercept term, β_i linear effect, β_{ii} squared effect and β_{ij} interaction term. Design expert software was performed for solving the regression equation and analysis of the response surface contour plot (Zheng et al., 2008).

Table 1. Range and Levels of variables

Variables		Range and Levels				
		-2	-1	0	1	2
X1	K_2HPO_4 (g/l)	5	7	9	11	13
X2	$NaNO_3$ (g/l)	10	13	16	19	22

Identification of strain by 16s rRNA gene sequences: DNA was isolated from biofilm cells using the protocol followed by Tillett and Neilan, (2000) with some minor modifications. 50 µl of TER buffer (10 Mm Tris HCl, pH 7.4; 1 mM EDTA pH 8; 100 µg/ml RNase A) was added into tube containing cell pellets. 750 µl freshly prepared XS buffer (1% Potassium ethyl Xanthogenate, 100 mM Tris HCl pH 7.4, 20 mM EDTA pH 8; 1% SDS; 800 mM ammonium acetate) and 10 µl RNase A were added and mixed by pipetting. After proper mixing, the solution tube was incubated at 70 ^{0}C for 60 min. After incubation, tube was kept on ice for 30 min. Cell debris was removed by centrifugation at 14,000 rpm for 10 min. DNA was precipitated in supernatant by adding chilled ethanol. The DNA pellet was obtained by centrifuging at 12,000 rpm for 10 min. DNA pellet was washed with 70% ethanol and air dried. Then, TE buffer was added (Tris HCl 10 mM and EDTA 1 mM) to dissolve the pellet.

Amplification process was carried out by primers DNM1 F 5'GAAAGCCTGACGGAGCAATA 3' and DNM1 R 5' CGGGACTTAACCCAACATCT 3'. PCR reaction mixture (20 µl) was prepared by mixing 10 µl Dream Taq master mix, 0.5 µl forward and reverse primers from 1:10 stock, 1 µl template DNA and 8 µl MilliQ water. 35 PCR cycles were performed, initial denaturation at 94 ^{o}C for 4mins, denaturation at 94^o C for 30 secs, elongation at 55 ^{o}C for 45 secs, extension at 72 ^{o}C for 2 min and final extension 7mins at 72 ^{o}C. DNA purification was carried out by GeneJET Gel Extraction (Thermo scientific). The purified DNA amplicon was subjected to Sanger sequencing. The identity of the strain was confirmed by BLAST analysis of obtained sequences with NCBI database (https://blast.ncbi.nlm.nih.gov).

RESULT AND DISCUSSION

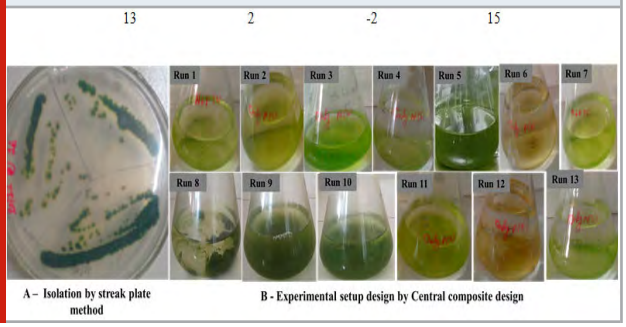
Isolation, Biofilm yield and Strain confirmation of

***Chroococcidiopsis* sp. by 16s rRNA gene sequences:** *Chroococcidiopsis* sp. isolation was done through streak plate method and individual colonies were observed on agar plates (Fig 1). The BLAST analysis of sequence data revealed the identity of the isolated organism as *C. cubana*. The sequence was deposited in NCBI GenBank data base with accession number MN950976. The biofilm yield of single colony grown in control and experimental flasks for 10 days were recorded (Table 2). Except run 4, the biofilm yield of all experimental flasks were significantly higher than of control.

Table 2. Experimental design with experimental yield (at 10thday)

Runs	X_1 (B)	X_2 (A)	Biofilm yield(g/l)
1	-2	0	10
2	2	0	18
3	0	0	20
4	-1	1	05
5	0	0	20
6	0	2	10
7	-1	-1	16
8	0	0	20
9	0	0	20
10	0	0	20
11	1	1	19
12	1	-1	12
13	2	-2	15

Figure 1: Isolation and experimental set up of CCD in lab



ANOVA for Response surface model: The effect of two independent variables were studied on one dependent response. The results of the second order response surface model for increase yield based on analysis of variance (ANOVA) is given in table 3. Regression equation of yield having empirical function of test variables in coded unit is shown in equation 3:

$$\hat{Y}_1 = 20 + 2.66X_1 - 1.38X_2 + 4.50\,X_1X_2 - 3.06\,X_1^2 - 3.81\,X_2^2$$

Where $\hat{Y}_1$ is the predicted yield, X1 is K_2HPO_4 and X_2 is $NaNO_3$.

ANOVA uses F – statistic to test the equality of means. F – test had very low probability value [(Prob>F) < 0.0001]. Hence, this model was highly significant for this experiment (Zheng et al., 2008). Goodness of fit of model was determined by determination coefficient (R^2). Determination coefficient, (R^2) value was 0.99 owing to which 99 % sample variation was qualified the variables, only 1% of the total variance could not be explained by this model. Adjusted determination coefficient also had similar value. Hence, the significance of the model was confirmed at required confidence level. Standard deviation (SD) was 0.47. Smaller the value of standard deviation, more precise the data. Because larger value of SD increases the acceptable range within the deviation. Coefficient of variation or pure error was 2.95%. Pure error lower than 5% considered to be an acceptable range of error (Box et al., 1978). Moreover, adequate precision had a value of 48.72. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable (Ahmadkhaniha et al., 2015). Because the model has a high signal it could be a so it's more reliable for optimization.

Table 3. ANOVA for response surface quadratic model

Source	Sum of Square	Degree of freedom	Mean Square	F – value	Probe>F (p value)
Model	300.79	5	60.16	277.10	<0.0001
Residual	1.52	7	0.22		
Lack of fit	1.52	3	0.51		
Pure error	0.00	4	0.00		
Total	302.31	12			

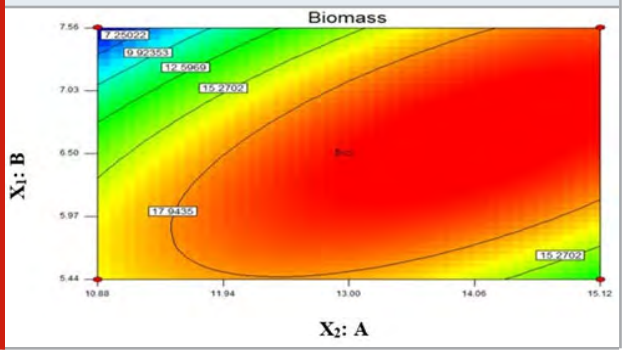
Standard deviation. = 0.47; Coefficient of variation = 2.95%; Mean = 15.77; R^2 = 0.99; Adj. R^2 = 0.99; Pred. R^2 = 0.96

Coefficient estimation for model: The significance of coefficient was determined by t value and p value. Both these values were computed and mentioned in Table 4. Higher the value of t – test and smaller the p value, the results are more significant (Lee and Wang, 1997). Our model intercept had large t value suggesting this model is highly significant. In model, variable X_1 had small p value as compare to variable X_2. Hence variable A was more significant than variable B in this model. The 2D contour plot represents graphical view of the regression equation (Wang et al., 2007). It had shown the effect of both the variables on yield of biofilm. Based on centre point of the contour plot, the obtained value of variable A ($NaNO_3$) and variable B (K_2HPO_4) were 13 and 6.5 respectively (Fig 2). Hence, both these values were found to be optimal for biofilm yield in 10 days. For optimization, the Design Expert 7.0.2 software suggested 0.95 and 1 as the two desirability standards. In current study, we proceeded for desirability 1 obtained value for A ($NaNO_3$) = 13 from lower limit 10 and upper limit 15 and value for B (K_2HPO_4) = 6.5 from lower limit 5 and upper limit 8. It was given 20 g/l yield in 10 days.

Table 4. Coefficient estimation of model

Factor	Coefficient	Std. error	Computed t – value	Computed p - value
Intercept	20	0.21	95.23	0.003
A	2.66	0.16	16.625	0.019
B	-1.38	0.16	-8.625	0.036
AB	4.50	0.23	19.57	0.016
A2	-3.06	0.18	-17.0	0.018
B2	-3.81	0.18	-21.0	0.015

Figure 2: 2D contour plot for *in vitro* biofilm yield of *Chroococcidiopsis cubana*



CONCLUSION

From the above results, it was concluded that variables A ($NaNO_3$ = 13 g/l) and B (K_2HPO_4 = 6.5 g/l) were giving similar results in experimental design data and for desirability of optimization. Hence, these values of the variables were used for upscaling the biofilm yield. The optimized model was successfully employed for *in vitro* cyanobacterial biofilm yield of *Chroococcidiopsis cubana*.

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Patterns of Root Resorption in Primary Mandibular Molars of Saudi Children

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ABSTRACT

The present study investigated patterns of root resorption in mandibular primary molars of Saudi children. The dental records of a randomly selected 460 Saudi children matched by age and gender who were treated in pediatric dentistry clinics at College of Dentistry, King Saud University in Riyadh, Saudi Arabia, were reviewed. Primary mandibular molar teeth were identified on the digital panoramic radiographs that were taken as part of the children's routine examinations. A trained and calibrated single examiner carried out all the examination procedures. The parameters such as symmetry and the level of mesial or the distal root resorption were investigated. The collected data were recorded on a special form and statistically computed. There were statistically significant differences in the distribution of the level of root resorption in the primary first and second molars; both the mesial and distal roots showed significantly less than a quarter root resorption followed by one-fourth to three-fourth levels of root resorption ($p<0.001$). The majority of the root resorption occurred symmetrically for the primary first (63.7%) and second (71.1%) molars. The resorption in the distal roots were comparatively more than the mesial roots in the primary first molars and vice versa in case of the primary second molars. A relatively high incidence of uneven level of root resorption was evident, more in the mandibular second primary molars with majority of the primary molars exhibiting less than a quarter root resorption. This requires close monitoring to ensure prevention of over-retention of primary molars. Though symmetrical resorption of mesial and distal roots of primary molars was observed in this study, variations does occur.

KEY WORDS: PRIMARY DENTITION, ROOT RESORPTION, MANDIBULAR MOLARS.

INTRODUCTION

Eruption of the primary dentition, their exfoliation as a result of root resorption, followed by eruption of permanent dentition is a complex, age specific event and occurs in an orderly sequence. (Pahkala et al., 1991) It is a critical milestone in the overall development of a child and is influenced by racial, ethnic, sexual, and individual factors. (Proffit et al., 2014) Initial resorption begins between one and three years after closure of the apices, and exfoliation follows by approximately three years. (McDonald et al., 2011).The roots of primary teeth resorb when the succeeding permanent tooth begins active eruption and the follicle comes into juxtaposition with the root surface.

Though the pressure of the erupting permanent tooth is believed to be a contributing factor initiating resorption,

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(Sahara, 2001) the presence of a permanent successor, however, is not a prerequisite for this process to occur. In the absence of a permanent successor, primary teeth do eventually resorb, although their exfoliation may be delayed than usual timings. (Cate, 1998, Obersztyn, 1963). There are several factors which affect the resorption of primary molar roots such as dental caries, congenital absence of permanent tooth bud and a number of pathological causes, (Sharma et al., 2016).

The development of the premolars commences between the diverging roots of the primary molars and the pattern of root resorption is affected by the position and size of the premolar follicles,(Fulton and Liversidge, 2016). Resorption of primary molar roots can be analogous, or perhaps irregular with one root considerably more resorbed than the other. Anatomically, the primary lower second molars' roots are very curved and divergent, and the inter-root distance is often greater than the size of the follicle of its successor. Unequal influences may be applied to the roots depending on the successor's position. This can explain the irregular root resorption detected in more than one third of all lower second molars at any given time after its initiation. On the other hand, the incidence of irregular root resorption is comparatively lower for the primary first molars and this is claimed to be due to the smaller difference between its inter-root distance and the size of the crown of its successor. (Prove et al., 1992a).

Longitudinal studies utilizing radiographs have been used to determine the extent and timing of resorption of the roots of primary teeth by comparing the resorbed root and the total root length, (Knott and O'Meara, 1967, Moorrees et al., 1963). However, the pattern of root resorption on radiographs is variable and can appear as a rounded or sharp apical margin, distinct or additional lateral thinning at initial stages of resorption and a horizontal or diagonal edge at advanced stages. (Fulton and Liversidge, 2016) Regional information regarding the patterns of root resorption of primary teeth are sparse. Hence, the present study investigated patterns of root resorption in mandibular primary molars of Saudi children.

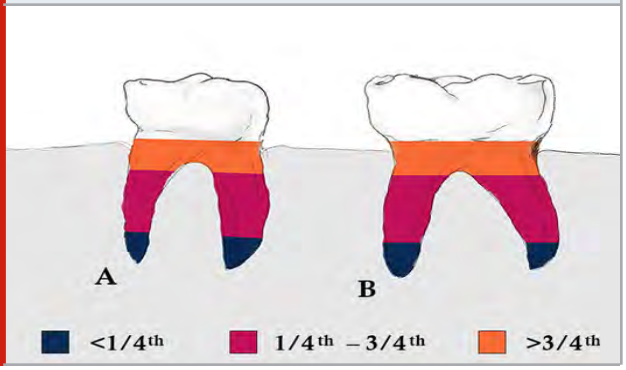
MATERIAL AND METHODS

The study protocol and consent form were approved by the Research and Ethical Committee of Human Studies at King Saud University, College of Dentistry Research Center (IR 0259). The dental records of a randomly selected 460 Saudi children matched by age and gender who were treated in pediatric dentistry clinics at College of Dentistry, King Saud University in Riyadh, Saudi Arabia, were reviewed. Children were selected for the study, if they were not diagnosed with any systemic diseases or taking any medications for chronic diseases.

Primary mandibular molar teeth were identified on the digital panoramic radiographs that were taken as part of the children's routine examinations and on which the teeth were present and fully visible. All radiographs were taken with Planmeca ProMax (Version 3.6.0, Helsinki, Finland), operating at 5 mA, 62 kVp. The radiographs were reviewed in identical conditions using Planmeca Romexis software (Version 4.0, Helsinki, Finland). A single examiner (WA) who was trained and calibrated (kappa=0.84) with a senior faculty in recording features of root resorption patterns carried out all the examination procedures. The intra-examiner reliability was established by re-examination of 40 radiographs on 2 different occasions with one week apart (Kappa=0.88). Radiographs were selected for the present study based on the following criteria :Clear image of the roots of the primary mandibular molars, clear image of the cementoenamel junction (CEJ), and no pulp treatment and no stainless-steel crowns.

The following parameters were investigated:1. The resorption of the mesial and distal roots (i.e. symmetrical or if either the mesial or the distal root were more resorbed); symmetry was defined if the line connecting the apices was parallel to a line connecting the CEJ at the mesial and distal surfaces.2. The level of resorption in the mesial and distal roots, according to the defined stages suggested by Moorrees et al.(1963) (less than a quarter, one-quarter to three-fourths, and greater than three-fourths of the root) as presented in Figure 1. The collected data were recorded on a special form and the Statistical Package for Social Science (SPSS V. 20, Chicago, IL, USA) was utilized for statistical computation. Data analysis included frequency distribution and chi-square was used to determine the significance of differences in level of root resorption with the level of significance set at $P<0.05$.

Figure 1: Index of root resorption (Source: Moorrees et al (Moorrees et al., 1963)). A. Mandibular first primary molar, B. Mandibular second primary molar.



RESULTS AND DISCUSSION

The age of the children ranged between 6-10 years, with a mean (±SD) age of 7.61 ± 1.24 years. Males and females were equally distributed (230 males, 230 females). There were statistically significant differences in the distribution of the level of root resorption in the primary first molars (Table 1) and primary second molars (Table 2). For both the molars, the mesial and distal roots showed significantly less than a quarter root resorption followed by one-fourth to three-fourth levels of root

resorption (p<0.001). For the primary first molars, the more number of mesial roots, irrespective of the side of the jaw, showed less than a quarter root resorption, when compared to the distal roots. For the primary right second molars, more number of the mesial roots showed less than a quarter root resorption than the distal roots on the right side. On the other hand, for the primary left second molars, more number of distal roots showed less than a quarter root resorption than the distal roots on the left side.

Table 1: Level of root resorption for the mandibular first primary molars.

Level of Root Resorption	Primary left first molar*		Primary right first molar*	
	Mesial n (%)	Distal n (%)	Mesial n (%)	Distal n (%)
< 1/4th	297(64.1)	249 (54.4)	306 (66.5)	247 (53.7)
1/4th – 3/4th	128(28.0)	167 (36.3)	115 (24.8)	173 (37.6)
> 3/4th	35 (7.9)	44 (9.3)	39 (8.7)	40 (8.7)
Total	460 (100)	460 (100)	460 (100)	460 (100)

* P<0.001, significantly different using Chi-square test

Table 3 shows the distributions of symmetry of root resorption of the mandibular primary molars which was found to be significantly different (p<0.001). The majority of the root resorption occurred symmetrically for the primary first molars (63.7%) and primary second molars (71.1%). The root resorption in the distal roots were comparatively more than the mesial roots in the primary first molars and vice versa in case of the primary second molars.This study investigated the patterns of root resorption of primary mandibular molars in a sample of 6-10-year-old Saudi children. Root resorption is a three-dimensional process and viewing this in two-dimensions certainly has some limitations. Resorption patterns in the bucco-lingual plane is less likely to appear well defined than in the mesio-distal plane, (Fulton and Liversidge, 2016).

Table 2: Level of root resorption for the mandibular second primary molars.

Level of Root Resorption	Primary left Second molar*		Primary right Second molar*	
	Mesial n (%)	Distal n (%)	Mesial n (%)	Distal n (%)
< 1/4th	307 (66.7)	334 (72.6)	319 (69.4)	247 (53.7)
1/4th – 3/4th	146 (31.7)	121 (26.1)	136 (29.3)	173 (37.6)
> 3/4th	7 (1.6)	5 (1.3)	5 (1.3)	40 (8.7)
Total	460 (100)	460 (100)	460 (100)	460 (100)

* P<0.001, significantly different using Chi-square test

There was symmetrical root resorption of the mesial and the distal roots in approximately 64 percent of the first primary molars and 71 percent of the second primary molars. This finding is higher in comparison to 5 to 12-year-old Israeli children where symmetrical root resorption of the mesial and the distal roots were found in approximately 41 percent of the cases.(Peretz et al., 2013) This finding is in contrast to previous studies where the distal roots(Peretz et al., 2013, Sharma et al., 2016) or the mesial roots(Moorrees et al., 1963) showed more resorption. The different study populations and variations in the study methodologies might have attributed to the contrasting findings. While Moorrees et al.(Moorrees et al., 1963) used lateral or oblique radiographs, Peretz et al.(2013) used peri-apical or bitewing radiographs and Sharma et al.(2016) used radiovisiographs. The present study utilized digital panoramic radiographs.

The position and size of the permanent tooth bud plays an imperative role in influencing asymmetrical pattern of resorption of primary tooth roots. This may be further justified when upper primary molars are taken into consideration, whereby the palatal root is often spared from resorption due to its highly divergent nature. Likewise, in anterior tooth, the completed crown of the permanent successor is found lingual to the primary tooth apex with its eruption in labial and incisal direction causing the resorption of the lingual surfaces of the apical third of the primary tooth root,(Prove et al., 1992b). In this study, both the mesial and distal roots of all the

mandibular primary molars showed significantly less than a quarter root resorption. This finding was more evident in the mandibular second primary molars as compared to the first primary molars. The mandibular primary second molars have roots that are curved and divergent and the crown width of the permanent successor is smaller than its inter root distance,(Nelson, 2014). Consequently, root portions of these teeth escape resorption and root fragments tend to remain which may affect the successors adversely, (Teague et al., 1999). Such uneven distribution of the level of root resorption warrants close monitoring and timely management to ensure no complications occurs due to over-retained primary teeth.

Table 3. Symmetry of root resorption for the mandibular primary molars.

Root Resorption	Primary First Molar* n (%)	Primary Second Molar* n (%)
> Distal Root	133 (28.9)	51 (11.1)
Symmetrical	293 (63.7)	327 (71.1)
> Mesial Root	34 (7.4)	82 (17.8)
Total	460 (100)	460 (100)

*P<0.001, significantly different using Chi-square test

In this study, the mandibular primary second molars showed greater propensity for symmetrical root resorption than the roots of the mandibular primary first molars. This finding is in contrast to the study involving 84 children between the age group of 7 to 10 years recruited from the University of Queensland dental school, (Prove et al., 1992b). A high incidence of ankylosis has been documented in both primary molars and this has contributed to the disturbances in the eruption and development of the premolars, (Tieu et al., 2013). However, the ankylosed molar often exfoliates spontaneously within six months; causing complications due to delayed exfoliation such as arch-length loss, occlusal disturbance, hooked roots or impaction of permanent successors,(Tieu et al., 2013).

In this study, certain limitations merits mention in order to improve on future studies. The cross-sectional design and the exclusion of various other parameters such as the position of erupting permanent successors, clinical status of the resorbing primary teeth and assessment of canine root resorption may be considered as major drawbacks. Further studies with a longitudinal design inclusive of the above-mentioned parameters may thus be conducted to gain a better understanding of the root resorption patterns of primary dentition.

CONCLUSION

Within the limitations of this study, a relatively high incidence of uneven level of root resorption was evident, more in the mandibular second primary molars with majority of the primary molars exhibiting less than a quarter root resorption. This requires close monitoring to ensure prevention of over-retention of primary molars. Though symmetrical resorption of mesial and distal roots of primary molars was observed in this study, variations does occur.

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Classification of Neurodegenerative Diseases Based on VGG 19 Deep Transfer Learning Architecture: A Deep Learning Approach

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ABSTRACT

This paper presents an automated system for the classification of two commonly diagnosed neurodegenerative diseases i.e. Alzheimer and Parkinsons, with the aid of deep transfer learning model Visual Geometry Group 19 (VGG19). As deep learning architectures are already very much used for the classification of various brain tumors and other types of cancers but not used much for the classification of Neurodegenerative diseases. There is an imperative need to have an automated system that can perform the classification among the Neurodegenerative diseases as the number of patients suffering from these disease are increasing. This system can even help the radiologists to enhance their diagnostic efficiency and accuracy of these Neurodegenerative diseases. In this paper, VGG19 deep transfer learning model is implemented, hyper tuned and trained over the Alzheimer and Parkinson disease (ADPP) dataset, which is developed using Alzheimer's disease Neuroimaging Initiative (ADNI) and Parkinson's Progression Markers Initiative (PPMI) databases for the multiclass classification of Alzheimer, healthy control and Parkinson disease. As there is no such system proposed yet to perform this degree of multiclass classification. So other popular deep transfer learning models like ResNet 50, Inception Net and VGG 16 are also implemented over the same ADPP dataset and their performance are compared with the proposed system based on VGG19 architecture. The proposed system based on VGG 19 outperforms the other three popular deep transfer learning models and delivers an average accuracy of 90% with 70/30 (training/validation split) and 93% with 80/20 (training/validation split) for the multiclass classification as Alzheimer disease, Healthy Control ADNI, Healthy Control PPMI and Parkinson disease. This work can be extended in future in order to propose an automated system for the multiclass brain structural disorders classification.

KEY WORDS: ALZHEIMER, INCEPTION NET, MPRAGE, PARKINSON, RESNET 50, VGG 16, VGG 19 ETC..

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INTRODUCTION

The two most common diagnosed neurodegenerative diseases are the Alzheimer's disease (AD) and Parkinsons disease (PD), which are diagnosed in old age people. Alzheimer disease is responsible for the dementia (Alzheimer's Association, 2017). A person suffering from Alzheimers, normally showcases the symptoms like difficulty in completing normal tasks, challenges

in planning, memory loss etc. This disease is a result of depletion of some particular nerve cells along with the existence of plaques (neuritic) just outside the neuron and aggregation of neurofibrillary tangles in the interior of neuron (McKhann et al., 1984). The neuritic and neurofibrillary proteins are whole sole responsible for the depletion of brain cells and destructing the functionality of brain cells. Thus the brain of people suffering from the advance stage of Alzheimer exhibits swelling and high shrinkage from cell loss. As AD is still incurable disease and its early diagnosis will be very useful for the good care of patients.

There are many ways adopted by the physician in order to diagnose the Alzheimer disease which involves problem solving, memory test, attention and the most effective is to take the brain scans of the patient in the form of either magnetic resonance imaging (MRI) or positron emission tomography (PET) or computed tomography (CT) scans. Mostly MRI scans are used by the physicians to diagnose Alzheimer disease. A number of deep learning-based approaches are proposed recently utilizing the MRI scans in order to automate the task of diagnosis and early prediction of Alzheimer disease (El-Sappagh et al., 2020; Feng et al., 2020; Rehman et al., 2020).

The Parkinson's disease was initially explained by James Parkinson, diagnosed in the older adults just like Alzheimer disease. The major symptoms of PD are muscle rigidity, tremor and slow movements (Borek et al., 2006). The various other indications of this disease are very low speech, face with no expressions, shaky handwriting, difficulty in standing from the chair etc. The PD is caused due to the deficiency of dopamine which acts as a neurotransmitter in the human brain. The loss of dopamine in the human brain is related to the depletion of neurons, which is a phenomenon occurs in old age people. The nature of this neurodegenerative disease in progressive which is similar to Alzheimer and hence early stage diagnosis of this disease will play an important role in the handling as well as caring of the patients (Ferreri et al., 2006). As the population of old age or senior citizens are increasing at a very rapid rate (Rehman et al., 2020).

So, there is a need of a systems that can diagnose these two neurodegenerative diseases at an early stage. As the MRI imaging exhibits accurate Neuro-anatomic biomarkers which plays an important role in the diagnosis of PD, so MRI scans are mostly used for the diagnosis of PD. Indeed MRI scans presents minute anatomical details related to the subcortical structures of the human brain whose analysis can be done in order to diagnosis this PD at an early stage. As these MRI scans are bound to be very difficult to analyze and observe the heterogeneous attributes as well as intrinsic details of subcortical structures captured in these MRI scans with the help of human eye because of the three dimensional nature. Hence there is a need of an intelligent system to perform this high data computing and processing task (Bakator et al., 2018; Lundervold et al., 2019). Recently a number of deep learning based classification approaches are proposed which have delivered high classification accuracy for the Parkinson disease prediction and classification utilizing MRI scans (Gautam and Sharma, 2020; Chakraborty et al., 2020).

Machine learning is very successful especially in the automated classification of different objects and various types of brain as well as human disorders, which is already proved by the number of papers published each year. In the context of brain disorders, machine learning and deep learning are widely used for the classification of brain tumors, glioma classification etc. utilizing electronic medical records and medical imaging data. Computer aided diagnosis (CAD) systems proposed over the years for the classification and diagnosis of breast cancer, lung cancers, liver cancer etc. are the application of machine learning in the domain of medical imaging. The huge amount of research is already been carried out in order to propose various CAD systems, which could be used to automate the task of diagnosis of various types of disease utilizing the PET, CT, fMRI and Structural MRI scans etc.

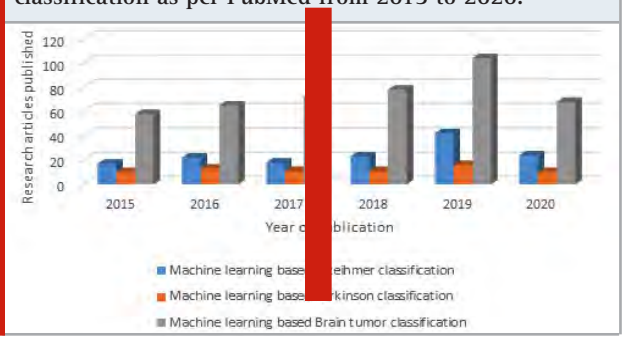
Now a days deep convolutional neural networks (CNN) are very popular and widely used by the researchers due to the high performance delivered by these models. As in deep learning models, there is no need of manual segmentation and feature extraction unlike conventional machine learning models, so these deep learning models are more popular and hence completely automate the task of either binary or multi class classification (Russakovsky et al., 2015). The deep transfer learning models like VGG 16, Inception Net etc. are widely used especially in the developments of CAD systems for the classification of various types of cancers and brain tumors (Mehrotra et al., 2020; Kaur and Gandhi 2020).

The literature review section given below simply highlights the very fact that there is no such unified approach proposed yet for the classification of both Alzheimer and Parkinson diseases. Although there are various deep learning based approaches proposed over the years for the individual classification of either Alzheimer or Parkinson disease. Another important fact proved by the literature review section that almost all these approaches based on machine learning and deep learning had used either the ADNI or the PPMI dataset for training and validation. The major contributions of this paper are as follows: A VGG-19 architecture based framework for the classification of two most common neurodegenerative disease used for the first time. The ADPP dataset, which is developed using the global datasets like ADNI and PPMI are used for training and validation purpose in order to showcase the applicability of this model in real time.

As there is no such unified framework or system for the classification of Alzheimer and Parkinson disease yet, so other popular deep transfer learning models like VGG-16, Res Net 50 and Inception Net are also implemented and trained over the same ADPP dataset in order to perform a genuine comparison with the proposed system

based on VGG-19. There is not much research carried out in the field of Alzheimer and Parkinson disease classification using machine learning as compare to the amount of research carried out for the brain tumor classification. But still a number of state of the art approaches based on traditional machine learning and deep learning models are presented in this section for both the neurodegenerative diseases. This fact is very well proved with the help of graph 1 given below which illustrates a comparison among the number of research papers published in the field of Alzheimer, Parkinson and Brain tumor classification based on machine learning.

Graph 1: The result of search for yearly rise in number of research papers related to the application of machine learning for the Alzheimer, Parkinson and Brain tumor classification as per PubMed from 2015 to 2020.



Initially Li et al. (2014) proposed an approach based on local binary pattern (LBP) and Support vector machine (SVM) classifier for the classification of Alzheimer. This machine learning based approach offers an accuracy of 83% on the MRI dataset prepared from the Alzheimer's disease Neuroimaging Initiative (ADNI) database. Then Dyrba et al. (2015) proposed another method based on Multi kernel SVM is proposed for the differentiation of Alzheimer patients from the normal ones. This approach uses graph-theoretical measures 'local clustering coefficient' methods for accurate segmentation from the diffusion tensor imaging (DTI) in order to calculate the grey matter (GM) volume. This approach offers an accuracy of 85% on the local dataset composed of MRI images of around 28 AD patients collected from the German Centre for Neurodegenerative Diseases (DZNE) Rostock database.

A SVM and multiple kernel learning (MKL) based approach was proposed for the correct classification of AD patients by Ni et al. (2016). The two above mentioned classifiers are trained with the help of linear, multi-fractal and mono fractal features extracted from the Resting estate functional MRI (rs-fMRI) scans. This approach offers an accuracy of 76% on the dataset prepared with the help of ADNI database. Another approach based on shape and volume based features along with the SVM classifier was proposed by Glozman et al. (2017) for the accurate classification of AD patients. This approach achieves an accuracy of 88% on the large size MRI dataset prepared with the help of ADNI database.

Valliani et al. (2017) used a deep residual convolutional neural networks (CNNs) pre-trained model known as the Resnet-18 for the classification and differentiation of Alzheimer patients from the normal ones. This Resnet-18 architecture performs learning of cross-domain features in order to optimize the interpretations of MRI AD images for the correct classification. This architecture achieves an accuracy of 81.3% on the dataset constructed with the help of ADNI database. In the same year Hon et al. (2017) came with an approach in which VGG-16 and Inception V4 transfer learning models are used for the classification of Alzheimer disease and delivers an accuracy of over 90% on the small size dataset prepared with the help of Open Access Series of Imaging Studies (OASIS) database. Another VGG-16 transfer learning based architecture is used by the Jain et al. (2018) for the correct classification of Alzheimer disease. This architecture delivers an accuracy of 95 % on the ADNI database based dataset.

Fulton et al. (2019) have proposed a Resnet-50 based approach, which delivers an accuracy of 98% on the dataset consist of cross-sectional and longitudinal MRI scans obtained from the OASIS database. A Resnet-34 deep transfer learning model based approach was also proposed by Talo et al. (2019), which performs multiclass classification for the Alzheimer disease. In this paper, data augmentation is also done in order to enhance the dataset for training. This approach based on Resnet-34 delivers an accuracy of 99% on a large size augmented MRI dataset. Similarly there are number of approaches based on the deep transfer learning models like VGG-16, Resnet 50, Alex net etc. which are used for the early classification of Alzheimer disease from the healthy ones and delivers accuracies in the range of 90-95%(Raghu et al., 2019; El-Sappagh et al., 2020; Feng et al., 2020; Rehman et al. 2020).

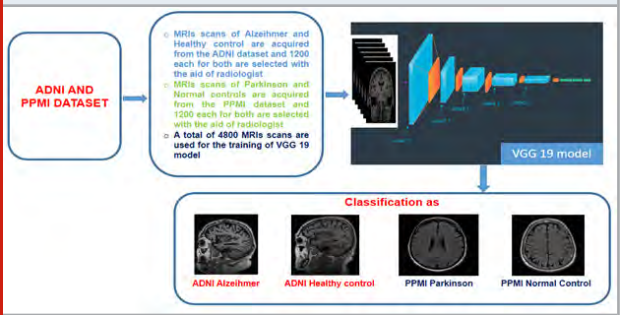
For the Parkinson disease detection and classification even less amount of research is carried out. Initially researchers are using traditional machine learning classifiers then employed deep learning models and recently deep transfer learning based approaches are used, which even delivering good results. A multi-kernel SVM based approach was proposed for thelassification of PD utilizing T1 weighted MRI scans all obtained from the Parkinson's Progression Markers Initiative (PPMI) repository and delivers an accuracy of 86% (Peng et al., 2017). Similarly another approach based on the SVM and random forest (RF) classifier was proposed which delivers an accuracy of 93% on the PPMI dataset (Amoroso et al., 2018). Then a transfer learned pre-trained AlexNet model based approach was proposed by Sivaranjini et al. (2019) in order to perform differentiation of PD patients from the normal ones. This Alexnet model is based on the proposed approach and delivers an accuracy of 88% on the dataset prepared from the PPMI repository.

Then Yagis et al. (2019) proposed another approach based on the two deep transfer learning models like VGG 16 and Resnet-50 for the correct classification of PD patients

from the healthy controls. Although VGG 16 and Resnet-50 delivers accuracy of more than 82 % on the large size dataset prepared with the aid of PPMI repository. Various papers for the automated classification of Parkinson disease have been published in the last two years primarily based on the 3D convolutional neural network (CNN) architecture (Chakraborty et al., 2020), Alex net architecture (Krizhevsky et al., 2012; Ortiz et al., 2019), conventional CNN model (Shah et al., 2018; Gautam and Sharma, 2020) providing accuracies in the range of 85-91% on the PPMI dataset.

The following points can be concluded from the brief literature review, which are as follows:1. There is a need of a unified automated system in order to classify both the Alzheimer and Parkinson disease as both are neurodegenerative diseases in real time, as there is no such system proposed yet for diagnosing the same.2. As ADNI and PPMI databases are the mostly used repositories consist of MRI images of both the neurodegenerative diseases. So these two databases are used to develop a common ADPP datasets consisting of Alzheimer and Parkinson diseases MRI scans.

Figure 1: Proposed automated system based on the VGG 19 architecture for the classification of Alzheimer and Parkinson neurodegenerative diseases.



MATERIAL AND METHODS

The proposed system based on VGG-19 deep transfer learning architecture consist of two major stages, first stage is all about downloading the MRI scans of Alzheimer and Parkinson's diseases from the ADNI and PPMI databases. Then conversion of MRI scans present in DICOM (Digital Imaging and Communications in Medicine) format into the PNG (Portable Network Graphics) format as this format offers lossless compression. Selection of informative MRI scans are carried out manually under the supervision of a radiologist, so that only the informative and correct MRI scans are present in the training and validation datasets. The second stage is all about customizing and training the VGG-19 with the help ADPP dataset. In the deep transfer learning VGG 19 architecture, the last three layers are modified in order to adapt to our problem domain and to perform correct multiclass classification into the Alzheimer, healthy control ADNI, Parkinson and healthy control PPMI classes. The proposed classification system is illustrated with the help of figure 1 given below:

ADPP Dataset description: ADPP is a balance dataset consisting a total of 4800 MRI scans of Alzheimer patients, healthy control scans from ADNI, Parkinson patients and healthy controls from PPMI is used in this paper for the training and validation/testing purpose. Around 1200 Magnetization Prepared Rapid Gradient Echo (MPRAGE) MRI scans each of Alzheimer patients and healthy control are taken from the ADNI database. Whereas 1200 axial T2 weighted MRI scans each of Parkinson's and healthy controls are taken from the PPMI database. Both these ADNI (www.adni.loni.usc.edu) and PPMI (Marek et al., 2011) datasets are publicly available datasets constantly used by the researchers working in the field of classification of different neurodegenerative diseases using machine learning.

Table 1. Demographic information of the ADPP dataset

Database	Groups	Number of objects	Sex	Age Group	MRI Modality	Number of MRI slices taken
ADNI	AD	50	24 females and 26 males	65-90	MP-RAGE	1200
	HC	50	35 females and 15 males	60-93	MP-RAGE	1200
PPMI	PD	50	20 females and 30 males	39-80	T2 weighted	1200
	HC	50	26 females and 24 males	31-80	T2 weighted	1200

From the ADNI database around 50 Alzheimer and 50 healthy control cases are downloaded in Dicom format. Similarly from the PPMI dataset around 50 Parkinson and 50 healthy control cases are downloaded. As the MRI scans are present in the Dicom format hence converted into the PNG format. The table 1 below simply illustrates the demographic information of the ADPP dataset used in this paper.

VGG 19 architecture: The VGG-19 architecture used in this paper simply belongs to the class of the visual geometry group network (VGGNet) developed by the

Oxford university (Tajbakhsh et al., 2016). The VGGNet is one of the popular models of deep transfer learning used for the image classification task. This VGGNet is trained on the ImageNet database (Yosinski et al., 2014; Russakovsky et al., 2015). The main reason behind using a VGG-19 architecture as it offers high accuracy, efficiency and adaptability to other classification problems. The architecture of VGG-19 used in this paper consist of total 19 layers which is divided into five blocks (Simonyan et al., 2014). Five max pooling functions are used to join these blocks. The input size is kept 224*224*3 and the size of the filter is kept very tiny i.e. 3*3 in each and every layers in order to handle the trainable parameters (Lee et al., 2018). The last three layers like Flatten, dropout and dense are added in our architecture in order to perform the multiclass classification and delivers the output. The architecture of the VGG-19 model used in this paper is illustrated with the help of figure 2 below.

RESULTS AND DISCUSSION

The simulation and experimentation of the proposed system based on VGG19 architecture and its comparison with the other three i.e. VGG 16, ResNet50 and Inception Net deep transfer learning models is performed using the Google Colaboratory (colab) platform powered by the NVidia Tesla T4 GPU. Whereas, Python 3.6 is used as an implementation programming language. The performance of the proposed system is evaluated and presented using the following statistical parameters or classification rates like Accuracy, Sensitivity, Specificity and F1-score, which are defined as:

$$\text{Accuracy} = \frac{TP+TN}{TP+TN+FP+FN};$$

$$\text{Precision} = \frac{TP}{TP+FP};$$

$$\text{Sensitivity} = \frac{TP}{TP+FN};$$

$$\text{F1 score} = \frac{2TP}{2TP+FP+FN}$$

Where FP= False positive, FN= False Negative, TP= True positive and TN= True Negative.

Figure 2: The VGG 19 architecture description along with the total number of parameters, trainable and non-trainable parameters information.

Layer (type)	Output Shape	Param #
input_9 (InputLayer)	(None, 224, 224, 3)	0
block1_conv1 (Conv2D)	(None, 224, 224, 64)	1792
block1_conv2 (Conv2D)	(None, 224, 224, 64)	36928
block1_pool (MaxPooling2D)	(None, 112, 112, 64)	0
block2_conv1 (Conv2D)	(None, 112, 112, 128)	73856
block2_conv2 (Conv2D)	(None, 112, 112, 128)	147584
block2_pool (MaxPooling2D)	(None, 56, 56, 128)	0
block3_conv1 (Conv2D)	(None, 56, 56, 256)	295168
block3_conv2 (Conv2D)	(None, 56, 56, 256)	590080
block3_conv3 (Conv2D)	(None, 56, 56, 256)	590080
block3_conv4 (Conv2D)	(None, 56, 56, 256)	590080
block3_pool (MaxPooling2D)	(None, 28, 28, 256)	0
block4_conv1 (Conv2D)	(None, 28, 28, 512)	1180160
block4_conv2 (Conv2D)	(None, 28, 28, 512)	2359808
block4_conv3 (Conv2D)	(None, 28, 28, 512)	2359808
block4_conv4 (Conv2D)	(None, 28, 28, 512)	2359808
block4_pool (MaxPooling2D)	(None, 14, 14, 512)	0
block5_conv1 (Conv2D)	(None, 14, 14, 512)	2359808
block5_conv2 (Conv2D)	(None, 14, 14, 512)	2359808
block5_conv3 (Conv2D)	(None, 14, 14, 512)	2359808
block5_conv4 (Conv2D)	(None, 14, 14, 512)	2359808
block5_pool (MaxPooling2D)	(None, 7, 7, 512)	0
flatten_7 (Flatten)	(None, 25088)	0
dropout_8 (Dropout)	(None, 25088)	0
dense_8 (Dense)	(None, 4)	100356

Trainable parameters	4819972
Non trainable parameters	15304768
Total parameters	20124740

Figure 3: Confusion matrices of the proposed system based on VGG19 (i) with 70/30 validation split; (ii) with 80/20 validation split

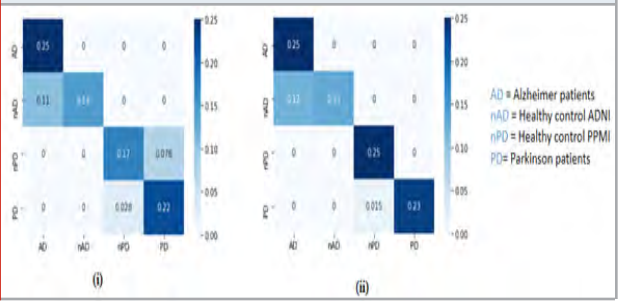
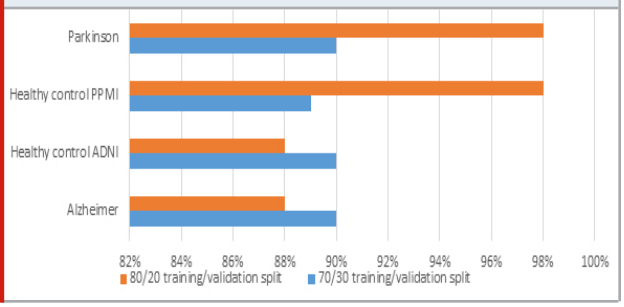


Figure 4: Accuracy comparison of the proposed automated system on the ADPP dataset with both 70/30 and 80/20 training/validation splits



The proposed system is evaluated in terms of above mentioned classification rates using two types of mostly used training/validation splits of the ADPP dataset as 70/30 and 80/20, which means 70, 80 % ADPP dataset is used for training and 30, 20 % for validation. The proposed system gives best results with 80/20 splits on the ADPP dataset. The classification rates are summarized in the table 2. The confusion matrices of the proposed

system is illustrated with the help of figure 3. The figure 4 simply presents the accuracy comparison of the proposed automated system with both 70/30 and 80/20 splits.

The proposed automated system based on VGG 19 is compared with some of the existing recent deep transfer learning models like VGG16, ResNet50 and Inception Net on the same dataset. The training and validation progress graph of VGG19, VGG16, ResNet 50 and Inception Net Model with split 70/30 are shown with the help of figures 6. All these models are implemented using the same programming language and same computing environment as of the proposed system based on VGG 19. Their brief comparison is illustrated with the help of table 3 and figure 5.

Table 2. Performance of the proposed VGG 19 based system on the ADPP dataset with 70/30 and 80/20 splits

Training and validation split percentage	Neurodegenerative diseases type classification	Accuracy	Precision	Sensitivity	F1 score
70/30 split	Alzheimer	90%	100%	70%	83%
	Healthy control ADNI	90%	58%	100%	73%
	Healthy control PPMI	89%	69%	86%	76%
	Parkinson	90%	89%	74%	81%
Average Accuracy, Precision, 90% sensitivity and F1 score		80%	83%	79%	
80/20 split	Alzheimer	88%	100%	67%	81%
	Healthy control ADNI	88%	52%	100%	68%
	Healthy control PPMI	98%	100%	94%	97%
	Parkinson	98%	94%	100%	97%
Average Accuracy, Precision, sensitivity and F1 score		93%	86%	91%	86%

Table 3. Performance comparison of the VGG19 based proposed system with the VGG16, ResNet50 and Inception Net based system on the ADPP dataset with 70/30 split.

Deep transfer learning model	Neurodegenerative diseases type classification	Accuracy	Precision	Sensitivity	F1 score
ResNet 50	Alzheimer	81%	64%	63%	64%
	Healthy control ADNI	82%	63%	64%	64%
	Healthy control PPMI	80%	42%	57%	51%
	Parkinson	80%	79%	67%	67%
Inception Net	Alzheimer	80%	99%	56%	71%
	Healthy control ADNI	80%	21%	97%	34%
	Healthy control PPMI	82%	77%	63%	69%
	Parkinson	83%	55%	70%	62%
VGG16	Alzheimer	88%	100%	69%	81%
	Healthy control ADNI	88%	54%	100%	70%
	Healthy control PPMI	89%	73%	80%	77%
	Parkinson	89%	82%	75%	78%
Proposed system based on VGG19	Alzheimer	90%	100%	70%	83%
	Healthy control ADNI	90%	58%	100%	73%
	Healthy control PPMI	89%	69%	86%	76%
	Parkinson	90%	89%	74%	81%

The proposed VGG19 based system is also compared with some of the state of the art approaches proposed in the recent years for either performing the Alzheimer classification or Parkinson classification on the ADNI or PPMI datasets. Their comparison is presented with the help of table 4 and 5.

Figure 5: Performance comparison graph for comparing the performance of VGG19 based proposed system with the VGG16, ResNet50 and Inception Net based system on the ADPP dataset with 70/30 split.

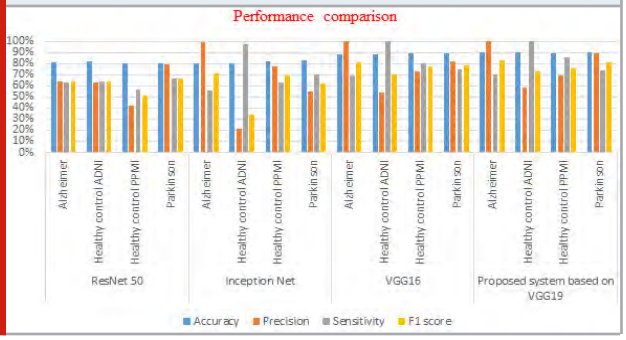


Figure 6: The training and validation progress graph of (i) proposed system based on VGG 19; (ii) VGG 16; (iii) ResNet 50; (iv) Inception Net on the ADPP dataset with 70/30 split.

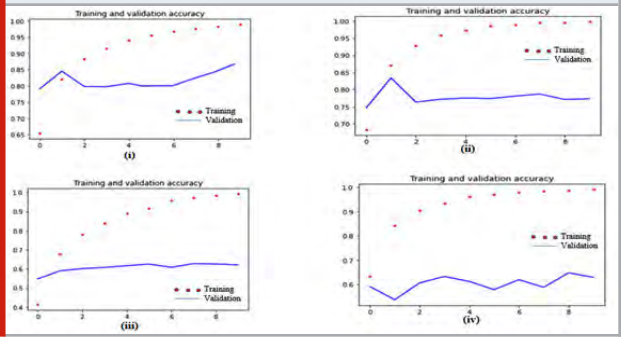


Table 4. Performance comparison of the proposed VGG19 based system with the existing approaches for the Alzheimer classification on the ADNI dataset.

Author and year	Machine learning classifier or deep learning model used	Accuracy	Precision	Sensitivity	F1 score
Li et al. and 2014	Support vector machine	83%	-	80.4%	-
Glozman et al. and 2017	Support vector machine	80.54%	-	70.59%	-
Valliani et al. and 2017	Residual Network 18 (ResNet18)	81.3%	-	-	-
Proposed one	VGG19	90%	80%	83%	79%

Table 5. Performance comparison of the proposed VGG19 based system with the existing approaches for the Parkinson's disease classification on the PPMI dataset.

Author and year	Machine learning classifier or deep learning model used	Accuracy	Precision	Sensitivity	F1 score
Peng et al. and 2017	Multi-kernel support vector machine (SVM)	85.78%	-	87.64%	-
Sivaranjini et al. and 2019	Alexnet	88.9%	-	89.3%	-
Yagis et al. and 2019	VGG16 and ResNet 50	82%	-	-	-
Proposed one	VGG19	90%	80%	83%	79%

CONCLUSION

The proposed automated system is a unified framework based on VGG19 for the classification of the two mostly diagnosed neurodegenerative diseases like Alzheimer and Parkinson. There are deep learning based systems or approaches for the classification of Alzheimer or Parkinson diseases, but there is not any system proposed yet which can perform the multiclass classification of these two neurodegenerative diseases. The proposed system is tested on the ADPP dataset which is developed using the global datasets like ADNI for Alzheimer and PPMI for Parkinson with the assistance of radiologist, so that only the informative MRI scans are used for training and validation. This systems delivers an encouraging average accuracy of above 90% for the multiclass classification, as this type of unified systems are proposed for the first time.

In order to present a genuine comparison, three mostly used deep transfer learning architectures like VGG16, ResNet50 and Inception Net are implemented and evaluated on the same ADPP dataset. The training and validation graph given in the result section, moreover proves that the proposed system based on VGG19 outperforms the VGG16, ResNet50 and Inception Net models in terms of accuracy and performance. In future, such unified systems can be enhanced and used in real time in order to assist the radiologist. More and more multiclass classification systems can be developed for the classification of other brain structural disorders like brain tumors, schizophrenia, dementia etc.

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Conflict of Interest: The authors have declared no conflict of interest.

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Applications of Artificial Intelligence in Clean Sustainable Energy System Support

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ABSTRACT

With increased competitiveness in energy generation industries, more resources are directed in optimizing plant operation in all aspects of the production, including fault detection and diagnosis, increase efficiency, forecasting the consumption and production. One of the most powerful tools in optimizing plant operation is artificial intelligence (AI). For the last few decades there has been major interest towards intelligent condition monitoring system (ICMS) application in power plant especially with AI development particularly in artificial neural network (ANN). It should be noted that the development of the energy industry is a step towards the development of other industries. That is why the transition to the digital industry is impossible without the digitalization and intellectualization of the energy industry. With massive possibility and room for improvement in AI, the inspiration for re-searching them are apparent, and literally, hundreds of pa-pers have been published, discussing the findings of hybrid AI for condition monitoring purposes. This paper attempts to discuss and review related work of AI and its applica-tion in energy industry. With regard to the energy indus-try, the integration of artificial intelligence in the industry will help optimize and improve efficiency in all aspects of the production, transmission and consumption of energy, fault detection and diagnosis, increase efficiency, forecast-ing the consumption and production. This note provides an overview of AI methods utilized for energy sector applica-tions, based on a systematic review of over 15 papers, 3 companies and commercial initiatives. The papers are clas-sified with regards to both the AI/ML algorithm(s) used and the application area in energy industry. We conclude the paper with a discussion of advantages and potential limitations of reviewed AI techniques for different tasks, and outlines directions for future research in this fast-growing area.

KEY WORDS: ARTIFICIAL INTELLIGENCE; DISTRIBUTED ENERGY RESOURCES, HOME ENERGY MANAGEMENT, MACHINE LEARNING.

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INTRODUCTION

The energy sector worldwide faces many challenges such as rais-ing demand, shortage of supply, environmental issue due to CO2 emis-sion. Many countries have already begun to use AI and the related technology in energy sector to allow better communication between smart grid, smart meter. AI can help improve power management effi-ciency, and use the renewable energy

sources more efficient, AI be-comes more and more important in energy industry and, indeed, it is having a great potential in future. With increased competitiveness in power generation industries, more resources are directed in optimizing plant operation, including fault detection and diagnosis. One of the most powerful tools in faults detection and diagnosis is artificial intelligence (AI). Faults should be detected early so correct mitigation measures can be taken, whilst false alarms should be eschewed to avoid un-necessary interruption and downtime. For the last few dec-ades there has been major interest towards intelligent condition moni-toring system (ICMS) application in power plant especially with AI development particularly in artificial neural network (ANN).

ANN is based on quite simple principles, but takes advantage of their mathe-matical nature, non-linear iteration to demonstrate powerful problem solving ability. With massive possibility and room for improvement in AI, the inspiration for researching them are apparent, and literally, hundreds of papers have been published, discussing the findings of hybrid AI for condition monitoring purposes. Alnaimi (2016) showed that ANNs are robust and reliable tools in energy sector application. They have been utilized to solve many operational problems, they presented a brief overview for applications of ANNs and GA for fault detection and diagnosis.

Sozontov (2019) dealt with the possibility of the implementation of artificial intelligence, which will improve the efficiency of national economy, including the energy industry. The paper presents the princi-ples of using neural networks and elements of artificial intelligence in the processes of production, transmission and consumption of electrici-ty. It has been revealed that the use of artificial intelligence in the elec-tric power industry will make it possible to minimize disruptions in power supply. It should be noted that the development of the electric power industry is a step towards the development of other industries. That is why the transition to the digital industry is impossible without the digitalization and intellectualization of the power industry. Increas-ing population worldwide demands more and more facilities, which in turn mandates the energy service providers to escalate their generation. Unfortunately, power generation globally is dominated by fossil fuels, which are the main contributor to CO2 in the atmosphere. Increasing CO2 emission threatens the world by global warming, as pointed out in the "World Energy Outlook 2019" (International Energy Agency, 2019).

To cope with global warming due to increasing CO2 emission from the traditional power system, governments around the world are encouraging renewable electric energy sources. For example, contrib-uting the green energy, motivated by declining capital costs and the government tax benefits, the United States added 72 gigawatts (GW) of new wind and solar (photovoltaic) capacity between 2018 and 2021 (U.S. Energy Information Administration, 2019). Similar renewable sources addition is carrying out across the globe today.

Recently, Serban and Lytras (2020) have reported that the synergy between renewable energy (RE) and AI will change the energy sector and improve sustainability at national and global level. Using AI could increase the efficiency of the RE sector by detecting and predicting patterns, by performing specific tasks without explicit instruction from human, by optimizing the supply and enhancing decision-making. It will provide better insights on processes due to speed forecasting and smart links between vital components as result of rapid development of technologies incorporating AI, (Lytras, 2017; Lytras, 2018; Visvizi, 2016). AI methods can be used to tackle various challenges, ranging from selecting the optimal set of consumers to respond, learning their attributes and preferences, dynamic pricing, scheduling and control of devices, learning how to incentivize participants in the DR schemes and how to reward them in a fair and economically efficient way (Antono-poulos, 2020).

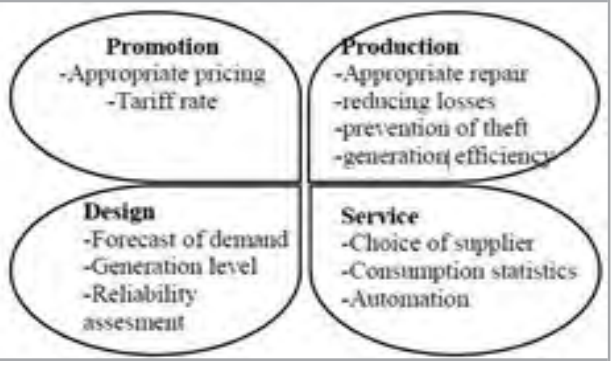
As nuclear power plants around the world reach their natural end-of-life, decommissioning plants – including Sellafield in Cumbria, Chernobyl in the Ukraine and Fukushima in Japan – offers a market opportunity for bespoke AI-enabled robots. Recent disaster situations such as Fukushima have shown the crucial importance of robotics tech-nology for clean-up and decommissioning of nuclear waste, monitoring and intervention, which is missing up to date, this is making AI work even more vital for energy sector (University of Lincoln, 2018). The application of AI technologies, particularly expert systems, to control room activities in a nuclear power plant can reduce operator error and improve plant safety and reliability. They constitute only a small fraction of those being developed, although few systems are actually in use in nuclear plants today.

RESULT AND DISCUSSION

There is no universally accepted definition for artificial intelli-gence, in this regard, by artificial intelligence we mean a machine sys-tem capable of learning, using objective knowledge and experience, solving creative problems like the human brain and inventive tasks without going through options, build strategies and apply abstract concepts (Losev, 2018). Figure 1 shows the concept of AI where it is a main branch of computer science that aims to create intelligent ma-chine. AI has become an essential part of technology industry. Ma-chine learning (ML) is a core of AI. ML uses data to train algorithms and gives computer system the ability to take decision and improve performance. Support vector machine (SVM), computer vision (CV) is type of ML. Deep learning (DL) is the most advance type of machine learning, Artificial Neural Network (ANN) is type of deep machine learning. In these days, AI has become an essential part in industry and life: Technology, manufacturing, banking, power station, electricity, energy, etc.

Figure 1: AI Approaches

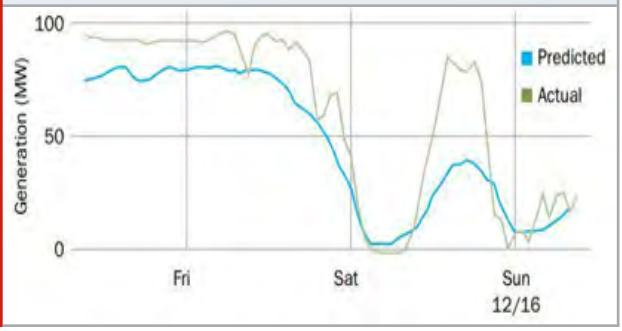
Figure 2: Tasks of artificial intelligence in Energy industry



The main tasks solved by artificial intelligence are presented in Figure 2.

At the design level, issues are being addressed to improve en-ergy demand fore-casting, as well as generation level, assessment of the reliability of power generating equipment, automation of relay protection systems and automation with increasing load on power plants. While at the production level, optimization of pre-ventive maintenance of equipment, increase of efficiency of gen-eration, reduction of losses and prevention of thefts of energy re-sources. For the promotion level, optimization of price depending on the time of year and day, and the tariff dynamics is also determined and justified.

Figure 3: Example of DeepMind Predictions vs. Actual in De-cember 2018. Source: Witherspoon, Sims and Will Fadrhonc. 2019. "Machine Learning Can Boost the Value of Wind Energy."



From the aspect of the service level, questions are solved on the automatic selection of the most prof-itable supplier, detailed statistics on energy consumption and en-ergy re-sources are provided, automated customer service is pro-vided, and energy consumption issues are optimized taking into account customer habits and behavior. Figure 3 shows the DeepMind System predicts energy output 36 hours ahead using neural networks, and recommends how to create optimal com-mitment on the grid, At General Motors, using artificial intelli-gence algorithms made it possible to increase the efficiency of wind turbines by 5%, while maintenance costs were reduced by 20% (Losev, 2019).

An interesting application of artificial intelligence methods was reported by the German company Schleswig-Holstein Netz AG, which operates electrical networks in the federal state of Schleswig-Holstein. Here, the self-learning network is used to determine the locations of the alleged damage. As initial data, this network uses information on per-formance of components of electrical networks and the repairs carried out, as well as information on loads and weather conditions. The Amer-ican company Air Fusion, which uses unmanned aerial vehicles to study the state of high-voltage power lines and wind turbines, uses software with artificial intelligence algorithms to process monitoring results.

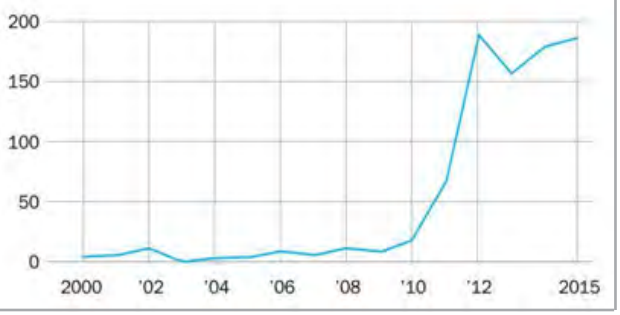
The neural network helps to solve the pattern recognition problem, for which thousands of images of damaged wind turbines are loaded into the program during the training process. Nuclear energy, which is one of the high-tech industries where developed countries retain its leading position. If we consider the introduction of artificial intelligence in this industry, then it will help solve a number of problems (Table 1).Any Artificial Intelligence is only as smart as its data. This is one of the biggest sticking points. The topics of data protection and data security are some of the greatest weak points for the use of Artificial Intelligence (Figure 4). Those who are connected digitally and intelligently reveal a lot about themselves and the system be-comes vulnerable to cyberattacks.

In 2018, the German Federal Office for Security (BSI) observed that the number of cyberattacks on critical infrastructure tripled in comparison to the previous year. Energy supply and the entire energy system are part of this critical infrastructure. This is why cyberse-curity is becoming more and more important today and in the future in order to protect the highly networked power grid from attacks and data theft from the outside. There are already strict security require-ments for participants in the electricity market in the area of data protection and data security, though. Contrary to the widespread opinion that AI makes the power grid less secure, AI can make an important contribution in the fight against cyberattacks. It can quick-ly check large amounts of data and thus detect deviations. AI can also draw conclusions from past cyberattacks. Machine Learning has already achieved great success in this area, for example in the detec-tion and defense of Trojans.

Table 1. The main results of the use of artificial intelligence in atomic energy.

No	Task	Task
1	Reactor safety	Identification location of defects
2	Nuclear power plant	Quick data flow analysis
3	Catastrophic risk management	Preventive emergency situations, reducing danger
4	Creating digital models of nuclear power plants	Ensuring the safety of nuclear power plants, the collection of all technical, technological and operational information
5	Artificial Intelligence and Automated Process Control System (APCS)	Appropriate resource allocation, increasing production efficiency Reduced development cycle,
6	Optimization of design processes	cost reduction. Solving complex problems
7	Advancement of science	Fast processing of data from scientific experiments and their further application
8	Technological process	Accelerate the pace of innovation and productivity
9	Cybersecurity	Finding vulnerabilities, writing codes and machine algorithms

Figure 4: Cyber Vulnerabilities. Source: Kaspersky Labs. Fickling, David. 2019. "Cyberattacks Make Smart Grids Look Pretty Dumb." Bloomberg.com, June 17, 2019. https://www.bloomberg.com/opinion/articles/2019-06-17/argentina-blaming-hackers-for-outage-makes-smart-grids-look-dumb



Many end users are critical of Artificial Intelligence, especially in relation to smart home technologies. This is understandable, because the data of the most private space that reveals a lot about its users is collected. Studies have shown that the biggest obstacle to the ac-ceptance of smart meters is fear of revealing private information without knowing exactly how it is used. These fears are justified, as there is still no regulation on how to handle this sensitive data, which is important for the electricity system of the future. Germany and the EU are trying to curb data access by private companies, as is hap-pening in the USA and China, for example. The EU Commission has therefore developed four basic ethical principles for AIs: AI should respect human autonomy, avoid social harm, be fair, and be explain-able. In order to give the energy industry and in particular end con-sumers more confidence in the AI, AI offers a multitude of suitable application scenarios that will support the energy transition and a climate-friendly energy system. It will be crucial, however, to protect user data and make the use of AI transparent and comprehensible (Vähäkainu, 2019).

CONCLUSION AND FUTURE WORK

With regard to the energy industry, the integration of artificial intelli-gence in the industry will help optimize and improve efficiency in all aspects of the production, transmission and consumption of energy, fault detection and diagnosis, increase efficiency, forecasting the con-sumption and production. It should be noted that the development of the energy industry is a step towards the development of other indus-tries. That is why the transition to the digital industry is impossible without the digitalization and intellectualization of the energy industry. It has been revealed that the use of artificial intelligence in the energy industry will make it possible to minimize disruptions in power supply. The topics of data protection and data security are some of the greatest weak points for the use of Artificial Intelligence. It is clear that the fu-ture lies in AI and furthermore, the capability of AI to revolutionize the energy sector must also not be doubted. AI can increase the efficiency, speed and security of energy consumption and generation and could lead the constant transitions in this sector to meet the changing climate needs.

But it also goes without saying that even this "intelligent tech-nology" has its own shortcomings which needs to be taken care of be-fore we can embrace it with open hands. For those looking to make a difference in shaping the future of society, the interface between AI and energy is a great place to start. Technological innovation is drastically changing the way we think about these two industries and their integration is in its early stages. Their synergy may change the world like we never knew it, and they are primed for innovative thinkers to make their mark. The future work depends of recharging new algorithms of AI which booster the efficiency of energy industry: production, distribution, marketing etc. Also it is important to take care of personality securing: more investment, time should be devoted to the aspect of cyber-attack related to big data and AI. Security, risk assignment, and hazard management of AI application in energy sector should be taken very serious. These topics should take a big part in future research at different levels.

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Method Development, Validation and Stress Studies of Dapagliflozin and Metformin Hydrochloride Using Ultraviolet-Visible Spectroscopy in Bulk and Combined Pharmaceutical Formulations

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ABSTRACT

A new, simple, precise, accurate, reproducible and economic stability indicating UV spectroscopic method was developed and validated for simultaneous estimation of Dapagliflozin and Metformin in pure and combined pharmaceutical dosage form. The UV spectrophotometric estimation of Dapagliflozin and Metformin was determined using the Q absorption ratio method at 222 nm and 232 nm respectively using water as diluent. The linearity ranges for Dapagliflozin and Metformin was 2 – 32 µg/ml and 1 – 20µg/ml respectively with their correlation coefficient values (R^2) 0.999. The percentage recovery at various concentration levels varied from 96.82 - 99.8 % for Dapagliflozin and 98.15 to 99.35 % for Metformin confirming that the method is accurate. LOD and LOQ for Dapagliflozin was found to be 0.0241µg/ml and 0.0293 µg/ml and for Metformin 0.0732 µg/ml and 0.0890 µg/ml. In the precision study, the% RSD value was found to be 0.1845 % and 0.2052 % for Dapagliflozin and Metformin respectively. Degradation studies were performed, both the drugs were found to be degraded in acid by using Hydrochloric acid, in base using sodium hydroxide solution, in peroxide using hydrogen peroxide solution, temperature and in light by exposing it to UV light in UV chamber. The results for estimation of Dapagliflozin and Metformin Hydrochloride and validation parameters like accuracy, precision, ruggedness, linearity were studied for the method and were found to be within the limits. The developed method was free from the interferences due to excipients present in the formulation and it can be used for routine quality control analysis.

KEY WORDS: DAPAGLIFLOZIN, DEGRADATION STUDIES, METFORMIN, Q ABSORPTION RATIO METHOD, STABILITY..

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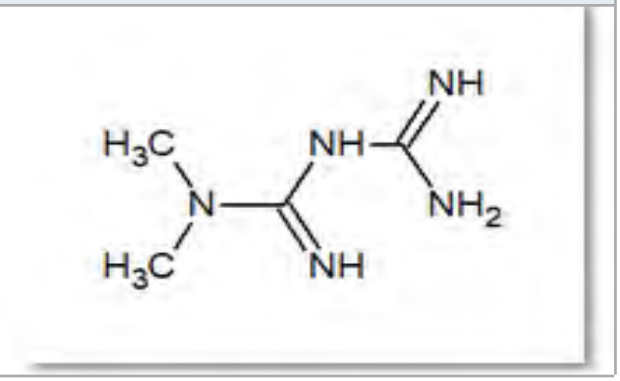
INTRODUCTION

Metformin (MET) hydrochloride (Fig.1) which chemically known as (3-(diamino methylidene)-1, 1-dimethylguanidine; hydrochloride. It has molecular formula of C4H11N5 and molecular weight is 165.62 g/mol. Metformin is an oral anti-hyperglycaemic agent (Type 2 diabetes) belongs to class of biguanides and useful for treating non-insulin-dependent diabetes mellitus. It decreases blood sugar levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization. These effects

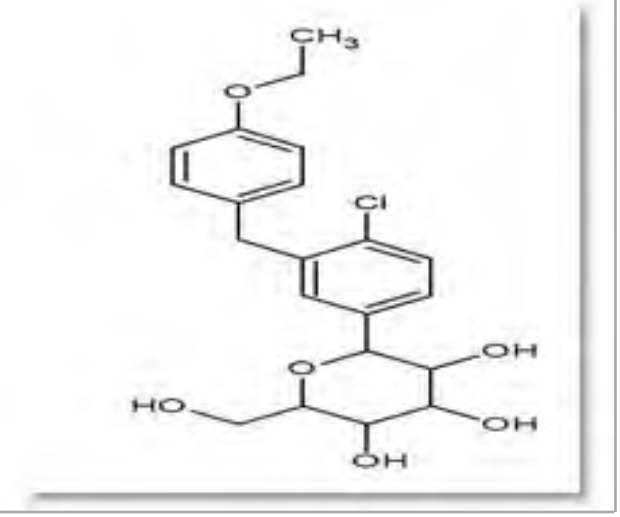
are mediated by the initial activation by AMP-activated protein kinase, a liver enzyme that plays an important role in insulin signalling, whole body energy balance, and the metabolism of glucose and fats (Mishra 2011; Urooj 2017).

Figure 1: Structure of Metformin.



Dapagliflozin (DAPA) (Fig. 2) is an antidiabetic drug. Its chemical name is (2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-(4-ethoxybenzy) phenyl]-6- (hydroxymethyl) tetrahydro-2H-pyran-3, 4, 5-triol. It acts as SGLT-2 inhibitor. Inhibition of this enzyme system reduces the rate of digestion of carbohydrates (Nalwade, 2012).

Figure 2: Structure of Dapagliflozin



A literature survey has revealed that only few articles on UV spectroscopic method for the simultaneous estimation of Dapagliflozin and Metformin (Mishra 2011; Jain 2015). The aim of our work was development of new, stability indicating UV method for determination ofDapagliflozin and Metformin HCL which possess the following advantages when compared to the already existing UV methods which is simple, cost-effective, and economic (Jain 2015). The main target for our new developed method is estimation of Dapagliflozin and Metformin HCL in the Pharmaceutical dosage forms.

MATERIAL AND METHODS

Spectrophotometric measurements were made in (ELICO) Double beam SL 210 UV-Visible spectrometer with 0.5 cm quartz cells. Drug Dapagliflozin and Metformin HCl were supplied as a gift sample from laboratory.Solubility of drugs 10mg of Dapagliflozin and Metformin HCL of each was weighed and checked in water, methanol and acetonitrile. Both the drugs were found to be soluble in water. For the selection of wavelength scan the standard solutions in UV spectrophotometer between 200 nm to 400 nm on spectrum mode, using water as a blank. The two drugs show λmax at 222 nm and 232 nm for Dapagliflozin and Metformin HCl respectively. 10 ppm solutions of both the drugs are scanned in the overlay mode to determine isosbestic point.

Figure 3: Spectrum of Dapagliflozin

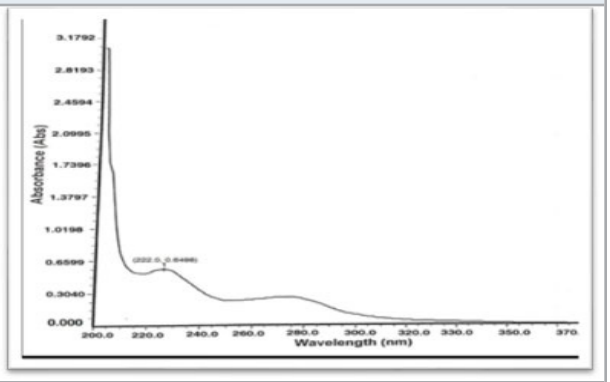
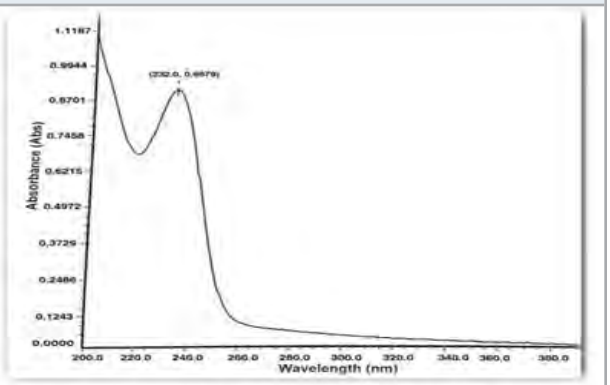


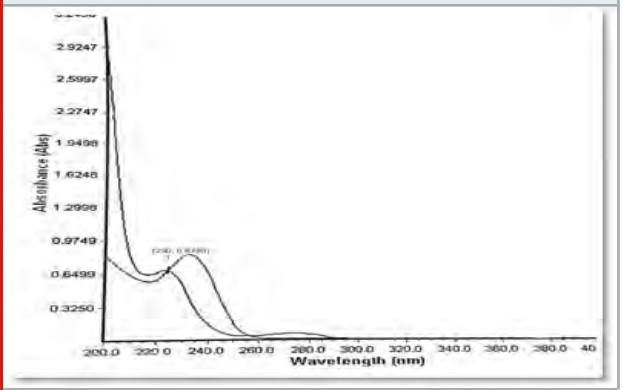
Figure 4: Spectrum of Metformin



Preparation of standard drug solution was done using 10 mg of Dapagliflozin and Metformin HCL was accurately weighed separately and dissolved in 5 ml diluent (Water), then transferred into a 10 ml volumetric flask, sonicated it for 10 min, finally, volume was made up to the mark with the same solvent to make 1000 µg/ml stock solution. From this 0.1 ml was again diluted to 10 ml to get a concentration of 10 µg/ml solution. It was scanned in UV range [200-400 nm] in 1.0 cm cell against solvent blank. The spectrum of drugs was recorded. After the study of spectrum of drugs, the λmax of Dapagliflozin

was found to be 222 nm and the absorbance was found to be 0.6498 and the λmax of Metformin was found to be 232 nm and the absorbance was found to be 0.9679. Isosbestic point was found to be at 230 nm and the absorbance was found to be 0.6789.

Figure 5: Overlay Spectrum



RESULTS AND DISCUSSION

Method validation is defined as the process that confirms the analytical procedure employed for a particular test is suitable for its intended use. Validation assures that a measurement process produces valid measurements. Results from method validation are used to judge the quality, reliability of analytical results. It is an integral part of any good analytical practice. The proposed method was validated for the parameters like linearity, accuracy and robustness as per ICH guideline (Parmar, Luhar and Narkhede, 2018). Accuracy indicates the deviation between the mean and true value. The accuracy is the closeness of agreement between the true value and test result. Accuracy was determined by means of recovery experiments. Solution containing known concentration of Dapagliflozin and Metformin HCL was used for this purpose. The accuracy was assessed from the test results as the percentage of the drug recovered by the assay at 3 levels (Patel, Chaudhary and Bhadani, 2017).

The linearity of an analytical method is its ability to elicit that test results are proportional to the concentration of drug in samples within a given range. Linearity of the method was determined by constructing calibration curves by taking. Standard solutions Dapagliflozin and Metformin HCL of different concentrations level (1mcg - 20mcg\ ml) and (2 - 36 mcg/ml) respectively were used for this purpose. Each absorbance was plotted against the concentrations to obtain the calibration curves and correlation coefficients. Characteristic parameters for regression equation ($y = mx^{+} c$) of the method were obtained by least squares treatment of the results and these parameters were used to confirm the good linearity of the method (Jani, Shah and Kapupara 2015).

Precision was estimated by studying repeatability by injecting 10 ppm concentration of Dapagliflozin and Metformin. The results were calculated as standard deviation, relative standard deviation (Heerspink, Zeeuw, and Wie, 2013).

Limit of Detection (LOD) is defined as the lowest level of concentration of analyte that can be detected, though not necessarily quantitated. It can be calculated from the below formula (Mishra, Soni and Nayak, 2011; Heerspink, Zeeuw, and Wie, 2013).

$LOD = 3.3\ \sigma/S$

Where,

σ = Standard deviation of the response,
S = Slope of calibration curve.

Limit of Quantization (LOQ) is defined as the lowest concentration of analyse that can be determined with acceptable accuracy and precision when the specified procedure is applied. It can be calculated from the below formula.

Table 1. Accuracy of Dapagliflozin

Accuracy Level	Sample Conc (Ppm)	Standard Conc (Ppm)	Drugconc (Ppm)	%Recovery	% Mean
50%	10	5	15	102.16%	99.8%
				99.11%	
				98.13%	
100%	10	10	20	98.55%	96.82%
				95.44%	
				96.46%	
150%	10	15	25	98.70%	99.31%
				99.53%	
				99.71%	

LOQ = 10 σ/S

Where,

σ = Standard deviation of the response,
S = Slope of calibration curve.

Robustness: It is the capacity of the method to remain unaffected by small but deliberate variations in method parameters. The analysis was performed by slightly changing the wavelength. To determine the robustness at +1 nm and -1nm from the fixed wave length. The results were calculated as % RSD Table 7 & 8 .10 ppm solutions of both the samples are used for the analysis (Nalwade, Reddy and Rao, 2012).

Assay of Tablets Formulation: For estimation of drugs in the commercial formulations, twenty tablets were weighed and average weight was calculated. The tablets were crushed and powdered in glass mortar. For the analysis of drugs, quantity of powder equivalent to 10 mg equivalent to Dapagliflozin and Metformin was transferred to 10 ml volumetric flask and dissolved in sufficient quantity of water. It was sonicated for 10 min and volume was made up to obtain a stock solution 1000µg/ml of Sample. Further dilutions were made from this stock solution to get 10µg/ml. The concentration of Dapagliflozin and Metformin was determined by measuring absorbance of sample solutions at 222 nm (λmax of Dapagliflozin) and232 nm (λmax of Metformin) using Q AbsorptionMethod. The results of analysis for the marketed tablet formulation (OXRAMET which contains 10 mg of Dapagliflozin and 500 mg of Metformin) are reported in Table 9. The amount of Dapagliflozin and Metformin was calculated using Q absorption ratio method given below (Sanagapati, Lakshmi and Reddy, 2014).

Forced Degradation: To assess the stability indicating property of the developed UV method stress studies were carried out under ICH recommended conditions.

Table 2. Accuracy of Metformin

Accuracy Level	Sample Conc (Ppm)	Standard Conc (Ppm)	Drug Conc (Ppm)	%Recovery	% Mean
50%	5	2.5	7.5	98.23%	99.35%
				99.20%	
				100.61%	
100%	5	5	10	98.03%	98.15%
				98.92%	
				97.49%	
150%	5	7.5	12.5	95.08%	98.85%
				94.39%	
				95.06%	

Acid Degradation: From the 100 ppm of drug solution, take 1 ml of the drug solution into 10 ml volumetric flask and 1 mL of 1 N HCL was added and was kept for 24 hours. After 24 hours neutralize with 1 ml of 1N NaOH room temperature, and further dilute with water to get concentration of 10 µg/mL and determine its absorbance (Satheeshkumar, Pradeepkumar and Shanthikumar 2014; Venkataraman and Manasa 2018).

Figure 5: Linearity of Metformin

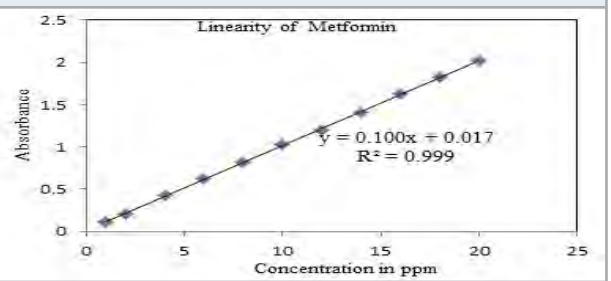


Table 3. Data of Linearity of Metformin

Concentration (ppm)	Absorbance at 232 nm
1	0.1132
2	0.2116
4	0.4215
6	0.6251
8	0.8243
10	1.025
12	1.2035
14	1.4098
16	1.6342
18	1.8213
20	2.0198

Figure 6: Linearity of Dapagliflozin

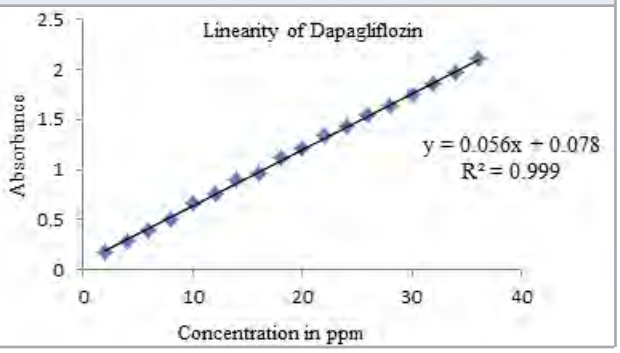


Table 4. Data of Linearity of Dapagliflozin

Concentration (ppm)	Absorbance at 222 nm
2	0.1725
4	0.2934
6	0.3942
8	0.5123
10	0.6563
12	0.7624
14	0.8946
16	0.9654
18	1.1145
20	1.2065
22	1.3458
24	1.4268
26	1.5368
28	1.6321
30	1.7524
32	1.8564
34	1.9751
36	2.1024

Table 5. Data for Precision

S.NO	Dapagliflozin	Metformin
1	0.5264	0.9963
2	0.5268	0.9953
3	0.5263	0.9965
4	0.5269	0.9946
5	0.5265	0.9998
6	0.5266	0.9996
Standard deviation	0.000787	0.00184
%RSD	0.1845	0.2052

Alkali Degradation: From the 100 ppm of drug solution, take 1 ml of the drug solution into 10 ml volumetric flask and 1 mL of 1N NaOH was added and was kept for 24 hours. After 24 hours neutralize with 1 ml of 1 N HCL room temperature, and further dilute with water to get concentration of 10 µg/mL and determine its absorbance (Venkataraman and Manasa 2018).

Table 6. Data for LOD & LOQ

DRUG NAME	LOD	LOQ
DAPAGLIFLOZIN	0.0241	0.0732
METFORMIN	0.0293	0.0890

Peroxide Degradation: From the 100 ppm of drug solution, take 1 ml of the drug solution into 10 ml volumetric flask and 1 mL of 30% Hydrogen peroxide solution was added and was kept for 24 hours. After 24 hours dilute with water to get concentration of 10 µg/mL and determine its absorbance.

Table 7. Evaluation data for Dapagliflozin Robustness study.

S.NO	221 nm	222 nm	223 nm
1	0.6479	0.6580	0.5499
2	0.6475	0.6582	0.5498
3	0.6478	0.6792	0.5588
Standard deviation	0.0002	0.0005	0.00516
%RSD	0.0008	0.0008	0.0079

Table 8. Evaluation data for Metformin Robustness study

S.NO	231 nm	232 nm	233 nm
1	0.9955	0.9952	0.9952
2	0.9950	0.9959	0.9955
3	0.9954	0.9958	0.9954
Standard deviation	0.00083	0.00038	0.00017
%RSD	0.00083	0.0381	00.0017

$$Cx = \frac{Qm - Qy}{Qx - Qy} * \frac{A_1}{ax_1}$$

$$Cy = \frac{Qm - Qx}{Qy - Qx} * \frac{A_1}{ay_1}$$

$$Q_m = \frac{\text{Absorbance of the sample solution at 222 nm (A2)}}{\text{Absorbance of the sample solution at 232 nm (A1)}}$$

$$Qx = \frac{\text{Absorptivity of Dapagliflozin at 222 nm}}{\text{Absorptivity of Dapagliflozin at 232 nm}}$$

$$Qy = \frac{\text{Absorptivity of Metformin at 222 nm}}{\text{Absorptivity of Metformin at 232 nm}}$$

Table 9. Evaluation data for Assay of tablets.

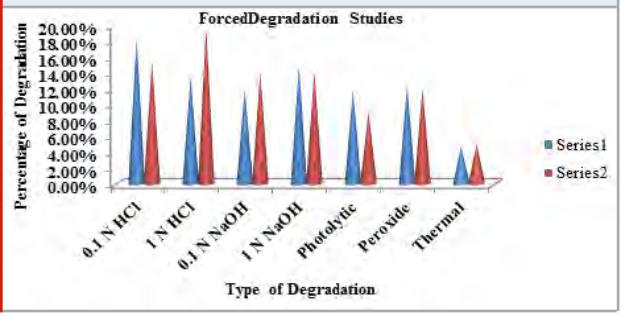
Drug Name	Label claim (mg)	Amount found
Dapagliflozin	10 mg	0.1957 µg/ml
Metformin	500 mg	9.7913 µg/ml

Table 10. Percentage of Degradation studies

S.No	Type of Degradation	Dapagliflozin (Series 1)	Metformin (Series 2)
1.	Acid Degradation (0.1N HCl)	18.35 %	15.32%
2.	Acid Degradation (1N HCl)	13.47%	19.64%
3.	Alkali Degradation (0.1N NaOH)	11.82%	13.98%
4.	Alkali Degradation (1N NaOH)	14.74%	14.04%
5.	Photolytic Degradation	11.68%	9.02%
6.	Peroxide Degradation	12.27%	11.95%
7.	Thermal Degradation	4.65%	5.02%

Photolytic Degradation: The bulk sample was exposed to UV light in UV chamber for 2 hrs by placing 10 mg of drugs in closed Petridish. The samples were appropriately diluted to get a final concentration of 10 µg/mL solution and were scannedover a range of 400 to 200 nm by placing respective solvents as blank (Swartz and Krull 2012; Urooj, Sundar and Vasanthi 2017).

Table 11. Degradation studies Graph



Thermal Degradation: The bulk sample was exposed to dry heat 80^{0}C in oven at for 2 hrs by placing 10 mg of drugs in closed petridish. The samples were appropriately diluted to get a final concentration of 10 µg/mL solution and were scanned over a range of 400 to 200 nm by placing the blank solutions and calculate the percentage of degradation (Urooj, Sundar and Vasanthi 2017).

CONCLUSION

From this validation study we can conclude that the developed UV method is accurate, rapid, precise, reproducible and inexpensive with acceptable correlation co-efficient, accuracy and robustness. The method is versatile for simultaneous determination of Dapagliflozin and Metformin with the use of low-cost reagents are the additional benefit of this method. So this method can be used for routine analysis in the quality control for determination of Dapagliflozin and Metformin.

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Therapeutic Effects of Metronidazole Benzoate in Combination with Melatonin in Diplomonad Parasite Infection on *Anabas testudineus*

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ABSTRACT

The diplomonad fish parasite of the Hexamitadae family frequently infects the fish *Anabas testudineus* during the warm season, leading to economic loss in the fish farming industry. Parasitic infection causes the generation of a large number of free radicals that promote oxidative stress in the fish body. This oxidative stress may cause direct tissue damage and affects the natural health condition of the fish population. Metronidazole benzoate (MB) is a widely accepted anti-protozoan drug, used to treat the protozoan infection in fish farming. The neurohormone melatonin is a potent free radical scavenger that is well known for its antioxidant, anti-inflammatory, and wound healing properties which can decrease the free-radical damage in liver tissue and reduce oxidative stress in fish body. The use of melatonin alone or in combination with other drugs to treat parasitic infection in fish has not been reported previously. Our current study shows a strong therapeutic potentiality of MB in combination with melatonin to treat the parasitic infection. The combination therapy caused a significant reduction of the lesion marks and the formation of new skin over the scar area. Complete recovery of liver histopathology was observed in the treated groups. The combination therapy also significantly improved blood cell counts to maintain body homeostasis recovery after infection. MB in combination with melatonin treatment gradually decreased the level of oxidative stress biomarker in parasite-infected fish. The level of antioxidative enzymes likes, CAT, SOD, and GPx was also significantly increased after treatment, which promotes the health recovery of infected fish. Thus, our study demonstrates that combination therapy of MB and melatonin effectively controls parasitic infection in *Anabas testudineus* which can be used to enhance the productivity in the fish farming industry.

KEY WORDS: AQUACULTURE, HEXAMITADAE, MELATONIN, METRONIDAZOLE BENZOATE (MB), OXIDATIVE STRESS.

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INTRODUCTION

The spread of infectious diseases in intensive fish farming is of major concern because it causes huge loss annually for the fish culture industry. Protozoan parasites are among the most common cause of fish disease in the culture system than any other fish parasites (Lom and Dyková, 1992; Abowei, Briyai, and Bassey, 2011). The diplomonad flagellate protozoa of the Hexamitadae family are generally intestinal parasites of fish and have

been associated with medium to severe mortalities in hatcheries (Roberts and Shepherd, 1974). The information about encystment in this species is scanty. It has been proposed that cyst-like structures confer long term survival to some protozoa species outside the host body. It becomes pathogenic when the number of parasites is very high in both salmonids and tropical aquarium fish (Lloyd and Williams, 2014).

Reports suggest that massive numbers of these protozoa cause high mortalities in hatchery fingerlings (Woo and Poynton, 1995; Uzmann, Paulik, and Hayduk, 2011). These parasites can potentially damage the host tissue and induce systemic infections. Some species are associated with the hole in the head diseases in cichlids. The typical characteristic of this disease is the appearance of parasite filled ulcerative lesions in the head region (Williams et al., 2011; Lloyd and Williams, 2014; Amesberger-Freitag et al., 2019). Histopathological analysis showed the formation of granulomatous tissue and lesions in the kidney, liver, and spleen of Atlantic salmon with systemic infections of flagellated protozoa. Reports suggested that flagellate parasites create problems with nutrition by consuming essential nutrients by disintegrating the epithelium of the intestine (Gratzek, 1988).

These parasitic infections are most frequently found in young carp and also some aquarium and various marine fish. The infected fish becomes weak, listless, anorexic, and emaciated so that the head appears large with respect to the body in the advanced stage of infection. Fishes show loss of equilibrium in highly infected conditions and they swim on their side (Lom and Dyková, 1992). Populations with acute infection exhibit high death rate over a very short period because of the rapid increase in the number of the parasite causes damage to the intestinal epithelium. The trout and other salmonids, when infected with these parasites, the effects commonly observed are anemia, weight loss, dark body color, intestinal anomaly, and excessive mucus secretion. Blood also ooze out from infected intestine because of intestinal hemorrhage and hepatic disintegration may also be found (Buchmann and Bresciani, 1997; Williams et al., 2011).

Severe parasitic infection in fish promotes the oxidative stress in the body of fish (Garcia Sampaio et al., 2008). Elevated oxidative stress can cause direct tissue damage and may decrease the performance and growth of a natural fish population (Stumbo, Goater, and Hontela, 2012). Metronidazole benzoate (MB) is the most common drug, widely used to treat the protozoan infection in fish farms and hatcheries. MB works better in partially reduced condition, and thus effective against anaerobic bacteria and protozoans. Melatonin is an indolamine present in the natural physiological system. It is well documented that exogenous melatonin can stimulate antioxidant enzymes and reduce the oxidative stress in fish (Mondal et al., 2017).

But there is no previous report on the application of melatonin in combination with other drugs to treat the parasite-infected fish or hole in the head disease in *Anabastes tudineus*. These reasons prompted us to test the therapeutic potentiality of the antioxidant melatonin alone or combined with MB to treat the parasitic infection in the fish population.

In our study, we assessed the toxicity and therapeutic efficacy of MB in combination with melatonin and demonstrated that combination therapy of MB with melatonin effectively controlled the parasitic infection and promoted recovery from the hole in the head disease in *Anabas testudineus*. MB is used as the most common drug used in the treatment of parasitic infection and melatonin is a stimulating agent of antioxidative enzymes. So, we observed the combined therapy significantly increased the antioxidative enzymes and reduced the oxidative stress of the parasitic infected fish body. After treatment by MB in combination with melatonin, the blood cell count was also increased compared to the diseased condition which supports the normal body homeostasis. Liver histopathology was also improved after treatment and new skin recovered the scar area. Therefore, MB in combination with melatonin can be used as a curing measure against parasitic infection to reduce the mortality and promote good health conditions of cultured fish.

MATERIAL AND METHODS

Chemicals: Metronidazole benzoate (MB) was purchased from J.B Chemicals and Pharmaceuticals Ltd (India). All other chemicals used in this study were purchased from Sigma–Aldrich Chemical Co (USA).

Animals: Adult specimens of *Anabas testudineus* L. (Climbing perch, Koi) (36 ± 2.20 g body weight, 10.1 ± 1.31 cm total length) a freshwater food fish present throughout the Indian subcontinent were collected from a local hatchery and were kept for 4 weeks in tap water in a large cemented tank for acclimatization. During this period fish became infected severely by the flagellated protozoan parasites. The infected fish developed a wound in the head region which subsequently developed a hole in the head (Figure. 3). Other physiological symptoms are observed like dark body colour, slow growth, faecal pseudocasts, and anorexia. The fishes were taken to the laboratory for further study and managed according to the standard protocol (Portaluppi, Smolensky, and Touitou, 2010).

LC_{50} estimation: Before testing the therapeutic potency, 24 h median lethal concentrations (96 h LC_{50}) of metronidazole benzoate was estimated by Probit analysis (Finney, 1971). Adult fishes were exposed to metronidazole benzoate treated water at different concentrations. Ten fishes were randomly assigned for each aquarium containing metronidazole benzoate treated water, prepared in tap water (having temperature 25 ±2.5°C, pH 7.1 ±0.3, free CO_2 24.7 ±2.4 mg l^{-1}, dissolved oxygen 5.2 ± 0.5 mg l-1, total alkalinity 132 ± 12.1 mg l^{-1} as $CaCO_3$, hardness 125 ± 3.2 mg l^{-1} as $CaCO_3$). Two different control groups were created, control A for natural healthy fish and control B for diseased fish.

The range-finding tests were performed to estimate the concentrations range of the experimental solution which varied from 100, 200, 300, 400, 500, and 600 mg l^{-1} metronidazole benzoate in tap water. The 24 h acute toxicity (confidence limits 95%) of the above composition to Anabas testudineus was estimated using statistical software, Finney Probit program. The percent fish mortality was subjected to statistical tests with the help of SPSS16 and Grpahpad prism to determine the significant variations among the mean mortality of test animals at different concentrations of the above composition. The protocols for all the biological assays and the physicochemical parameters of the test water were determined by following the methods of APHA (2012).

Experimental design: The experiments were performed in 5 glass aquaria filled with tap water. After confirming the LC_{50} value of metronidazole benzoate, we selected two doses below the LC_{50} value in comparison with two control groups and one group only for melatonin treatment. The fishes were not fed for 24 h before the commencement of the test. Identical groups of ten fishes were kept in five separate aquaria containing 6 l of plain tap water as control (negative control, containing healthy fish; 0.7% NaCl); diseased (positive control, containing infected fish; 0.7% NaCl); melatonin (100 pg l^{-1} of melatonin; 0.7% NaCl); experiment 1 (100 mg l^{-1} of metronidazole benzoate and 100 pg l^{-1} of melatonin; 0.7% NaCl) and experiment 2 (200 mg l^{-1} of metronidazole benzoate and 100 pg l-1 of melatonin; 0.7% NaCl). After 36 h of incubation in natural aerations, physiological observations were performed. Liver samples were collected after 3 days by sacrificing the fish to study the liver histopathology. Oxidative stress parameters and antioxidant activity were biochemically estimated.

Histopathology of liver tissue: To study the histopathology, liver specimens were collected from each fish and then fixed with 10% neutral buffered formalin. Tissue samples were processed routinely for paraffin sections of 4–5 µm thickness, stained with hematoxylin and eosin (Khalil et al., 2007).

Differential count of blood cells: The blood was used for the estimation of red blood cells or erythrocyte and white blood cells or leucocytes counts. Erythrocyte and leucocytes were counted by the method of Rusia and Sood (1992) using a haemocytometer.

Enzymatic antioxidative agents: Superoxide dismutase activity: Superoxide dismutase (SOD) activity was measured according to the protocol described previously (Ewing and Janero, 1995). 0.2 g of liver tissue sample was taken and homogenized in 2 ml cold homogenization buffer solution. The suspension was then centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant was collected for the enzyme assay. 100 µl of the extract was taken for analysis. Blank samples were covered to protect them from light whereas control samples were exposed to light. Then, they were illuminated for 10 minutes. The absorbance was measured at 560 nm with reference to the blank. One unit of SOD activity is defined as that amount of protein (in mg) causing a 50% inhibition of the photoreduction.

Catalase activity: The catalase (CAT) activity was estimated by spectrophotometric method (Aebi, 1984). 40 µl of the hepatic supernatant was taken in a cuvette and rapidly mixed with H_2O_2 phosphate buffer. Absorbance at 240 nm was measured using Beckman Coulter, DU 730, Life Science UV-VIS spectrophotometer up to 120 seconds at 15 seconds intervals.

Glutathione peroxidase (GPx) activity: GPx activity was measured by using the spectrophotometric method described by Castro (2008). Orthophenyl diphosphate (OPD) which is the substrate of GPx was used in this assay. Serial dilutions of OPD were made in phosphate citrate buffer (pH 5.0). 1 ml of each OPD serial dilution was mixed with 100 µl of hepatic supernatant sample and then 0.9 ml of 0.013% H_2O_2 was added and incubated at room temperature for 30 minutes. Absorbance was measured at 492 nm with reference to the blank. The change of absorbance value 1.0 under the assay condition is equivalent to the one unit of the enzyme action.

Determination of the level of malondialdehyde: The liver homogenates were centrifuged at 3000 g for 15 minutes and the supernatants were collected for the estimation of malondialdehyde (MDA) level by thiobarbituric acid (TBA) reactive assay with minor modifications (Draper and Hadley, 1990). Then 1 ml of the sample was taken and heated in a water bath at boiling temperature with TBA reagent (20% trichloroacetic acid, 0.5% TBA, and 2.5 N HCl; 2 ml) for 20 minutes. After cooling down the reaction mixture was centrifuged at 500 g for 10 minutes and the pellet was removed. The absorbance was measured at 532 nm. The MDA equivalents were calculated by using an extinction coefficient of 1.56×10^5/Mcm.

Statistical analysis: The data variable in a particular sampling period was expressed as mean SEM of the individuals (n =10). As all data sets passed the normality test ($p<0.05$), the differences in the values of each experimental group (natural control, diseased control, melatonin treated, and two different concentrations of metronidazole benzoate and melatonin treatment) were calculated by one-way ANOVA test. F-values indicated the significance. The means were compared by using a post hoc Duncan's multiplerange test, with $p< 0.05$ taken as the statistically significant threshold. In addition, a correlation coefficient test was performed to study the correlations between the profiles of different antioxidative agents, and MDA (any 2 variables at a time) in the liver of each control and experimental group. A linear regression analysis was performed for expressing the dependence of a response variable on an independent (predictor) variable. $p< 0.05$ level was considered as a statistically significant value in each case.

RESULTS AND DISCUSSION

Metronidazole benzoate is widely used in veterinary medicine as well as in aquaculture to prevent contamination by many virulent protozoan organisms. To investigate how metronidazole benzoate in combination with melatonin cause different degrees of stress, life parameters like acute toxicity, disease control, oxidative stress with antioxidant enzyme activities, and recovery from parasitic infection was assessed.

LC_{50} values of MB on Anabas testudineus: Before starting treatment, we estimated the LC_{50} dose of MB to determine the therapeutic dose. This one was the first attempt to determine the LC_{50} of this common medicine used in a wide range of species from mammals to fish. LC_{50} values for MB of 24 hours of incubation was 357.12 mg l^{-1} (Table 1, 2.). Probit mortality analysis is also shown in Figure 1; 2.

Figure 1: Percent Mortality of *Anabas testudineus* against different concentrations of metronidazole benzoate after 24 h of exposure. ($p < 0.05$).

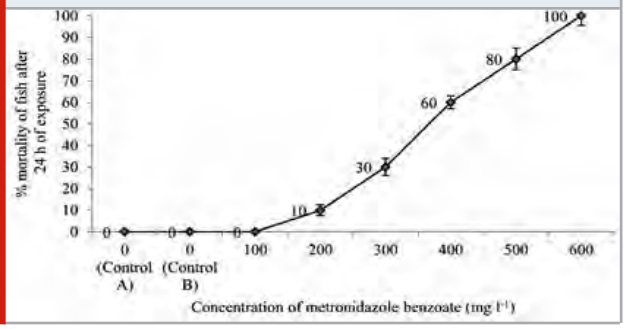
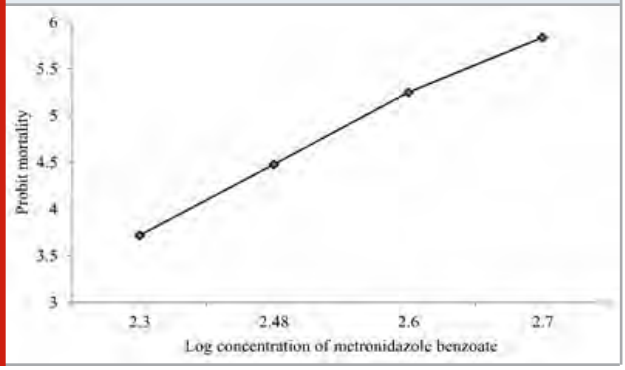


Figure 2: Probit mortality of *Anabas testudineus* against different log concentrations of metronidazole benzoate after 24 h of exposure. ($p < 0.05$).



Physical observations: We found that the MB in combination with melatonin repairs the wound created by the infection of the protozoan parasite very rapidly as soon as 48 to 72 hours. After 36 hours of metronidazole benzoate (different doses) in combination with melatonin treatment, we found a great reduction of the scar area and new skin reformed. After 72 hours, the scars disappeared and no lesion mark was visible. The fish became dark in color with the restoration of their healthy movement and balance (Figure. 3.).

Figure 3: Phenotypic observation of normal control, disease control and treated groups. Different combinations of treatment showing various levels of scar recovery in parasite-infected fish *Anabas testudineus*. Highest head scar recovery found in MB (200 mg l-1) in combination with melatonin treated group.

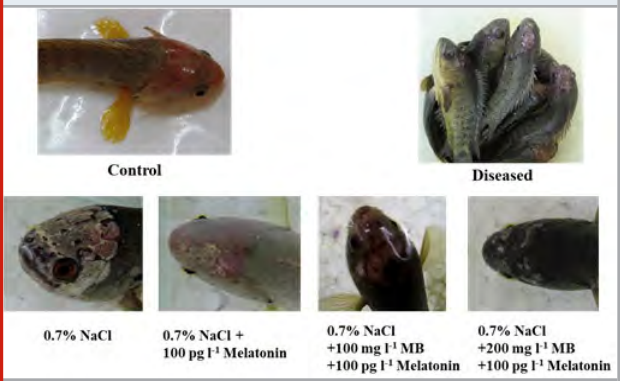
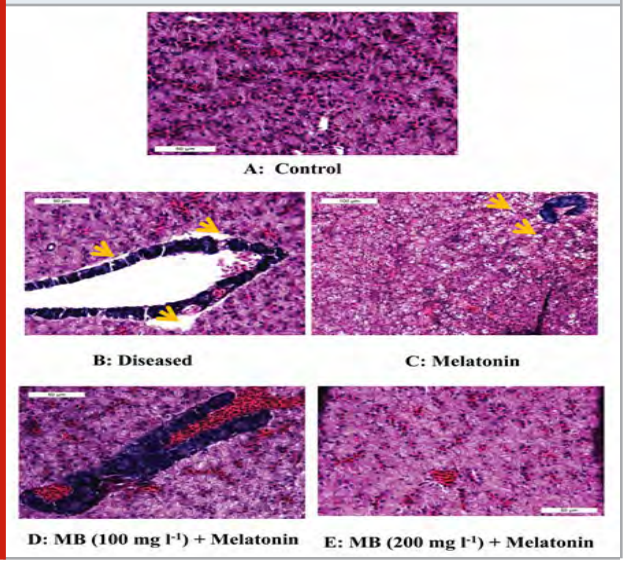


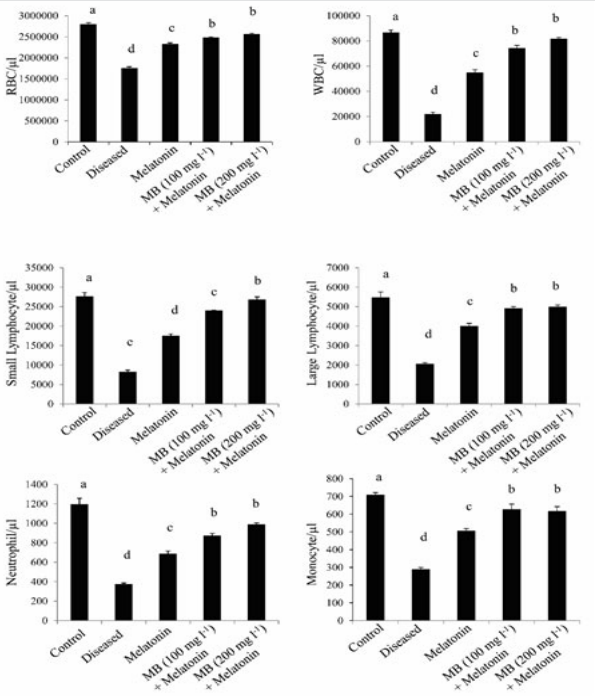
Figure 4: Histopathology of liver tissue from the control, diseased and treated groups. Photomicrograph of liver tissue (A) control showing normal histology, (B) diseased showing highly damaged condition like big space present between hepatic tissue and pancreas tissue and also hepato-pancreas showing vacuolation, (C) melatonin treated demonstrated slight recovery in tissue structure but some vacuolation also present, (D) MB (100mgl^{-1}) + melatonin and (E) MB (200mgl^{-1}) + melatonin liver of *Anabas testudineus* showing highly recovered structure like control. Pancreas (deep blue structure), vacuolation (yellow arrow), blood congestion (within the hepato-pancreas in image D.). Stained with H & E.



The present study provides strong evidence for MB in combination with melatonin having a good recovery capacity against the diplomonad parasite infection in the head region of the tropical fish Anabas testudineus. Before this study, no such reports were showing rapid recovery from a parasitic infection of the Hexamitadae

family in *A. testudineus* or any other fish species. MB can solely cure this protozoan infection but it usually takes a longer duration. On the other hand, we have seen that melatonin also has the curing capacity to some extent, but it was less effective than MB when it is applied alone. However, their combination is highly effective to cure. Higher concentration (but less than LC_{50}) of MB has better curing and repairing the property. This may help in reducing mortality in the hatchery or the stocking of fish.

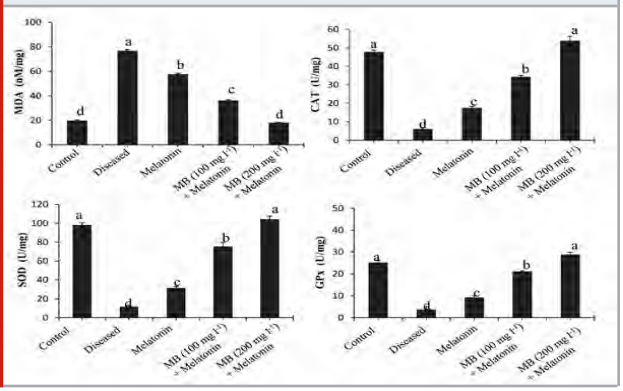
Figure 5: Differential blood cells counts in the control, diseased and treated groups. Histogram showing the mean values (±SEM, in vertical bars) of the differential count in blood cells of *Anabas testudineus* in different experimental groups following the control, diseased or melatonin solutions (melatonin treated at the dosage of 100 pg l^{-1}) and different doses of MB (100 or 200 mgl^{-1}) in combination with melatonin. The alphabets on the error bars indicate significant ($p < 0.05$) differences in the values of a particular variable after one-way ANOVA and Duncan's multiple range test (DMRT) thereafter. The same alphabets indicate no significant change in the values of a particular variable.



Histopathological observation of liver: The histopathological examination of all liver tissues collected from fish exposed to a different combination of MB and Melatonin showed a variation in structure. However, the most affected pathological lesions were observed in the diseased control group showed major alterations of architectures with hepatocyte degeneration and vascular dilation. Gradual improvements in the histopathology were observed in the only melatonin treatment group, but the highest recovery of the architecture of the liver was found in the combination of MB and melatonin treatment groups after 3 days (Figure. 4.).

Differential blood cell counts: The blood cell counts among different experimental groups showed significant variations. Red Blood Cells (RBC) count was significantly less in the diseased group whereas the highest RBC count was observed in the healthy control group and different treatment groups like only melatonin, 100 mg l^{-1} MB + melatonin, and 200 mg l^{-1} MB + melatonin, which showed a gradual increase in cell counts. This data indicates that heavy infection of this parasite causing nutritional deficiency, which resulted in less RBC count in the diseased fish (Gratzek, 1988). The increase in RBC counts may be due to the combined effect of MB and melatonin on hematopoiesis. Similar patterns were found in different White Blood Cell (WBC) count, showing least in disease control and highest in the healthy control and treated groups (Figure. 5). An increase in WBC gives an indication of immunostimulation which may help the fish to maintain body homeostasis recovery from infection. Further research is needed to prove this hypothesis.

Figure 6: Antioxidant enzyme activities of MDA, CAT, SOD and GPx in the hepatic tissue of control, diseased and treated groups. Diagrammatic presentation of the mean values (±SEM, in vertical bars) of the levels of oxidative stress marker MDA and different enzymatic antioxidant (CAT, SOD, and GPx) in the liver of *Anabas testudineus* in the different experimental group following control, diseased or melatonin solutions (melatonin treated at the dosage of 100 pg l^{-1}) and in different doses of MB (100 mg l^{-1} and 200 mg l^{-1}) in combination with melatonin. The alphabets on the error bars indicate significant ($p < 0.05$) differences in the values of a particular variable after one-way ANOVA and Duncan's multiple range test (DMRT) thereafter. The same alphabets indicate no significant change in the values of a particular variable. MDA, Malondialdehyde; CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase.



Malondialdehyde (MDA) level in the Liver: Parasitic infection can induce oxidative stress in the host tissue. Therefore, we found that the parasitic infection significantly increased lipid peroxidation which is considered as a marker of oxidative stress. Our experiment showed that high levels of lipid peroxidation product MDA present in the liver of diseased fish, which demonstrate that individuals may have a high level of oxidative stress due to parasitic infection.

Figure 7: Regression analysis between MDA concentrations and the values of CAT or SOD or GPx in the hepatic tissue of control, diseased and treated groups. Scatter plots representing the results of single regression analysis between hepatic MDA concentrations and the values of CAT or SOD or GPx in the liver of *Anabas testudineus* in the different experimental group following control, diseased, melatonin solutions (100 pg l^{-1}) or different doses of MB (100 mg l^{-1} and 200 mg l^{-1}) in combination with melatonine. R2 denotes goodness of fit. CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.

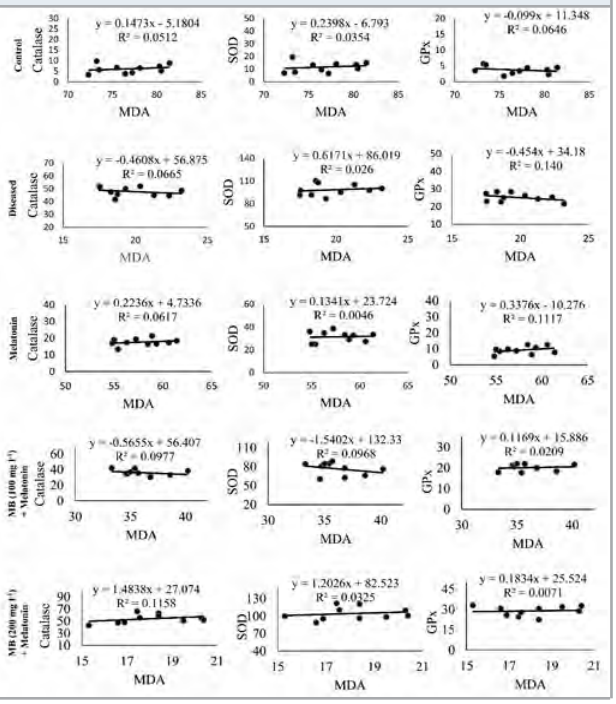


Table 1. Mean values of % mortality of *Anabas testudineus* exposed to different (0-600 mg l^{-1}) concentrations of metronidazole benzoate dissolved in 0.7% saline after 24 h of exposure. Mean values within columns indicated by different superscript letters (a-f) and are significantly different (one way ANOVA at 5% level).

Concentration (mg l-1)	% mortality of fish exposed to MB at 24 hour of exposure
0 (Control A)	0 ± 0[a]
0 (Control B)	0 ± 0[a]
100	0 ± 0[a]
200	10 ± 2.5[b]
300	30 ± 4[c]
400	60 ± 3[d]
500	80 ± 5[e]
600	100 ± 4.5[f]

The MDA level decreased gradually after treatment with only melatonin, 100 mg l^{-1} MB + melatonin, and 200 mg l^{-1} MB + melatonin groups (Figure. 6.). This result signifies that treatment with MB with melatonin decreased the oxidative stress in the liver tissue of fish (Mondal et al., 2017).

Table 2: LC value (LC_{50} with 95% confidence limits, regression equation, R2 and r values) of MB after 24 h of exposure of *Anabas testudineus*.

Lethal Concentration Values (mg l^{-1})						95% Fiducial Limits of LC_{50}		Probit Regression Equation Y=ax+b	R^2 Value	r value
LC_1	LC_{10}	LC_{30}	**LC_{50}**	LC_{70}	LC_{90}	Lower	Upper			
131.09	205.61	284.91	**357.12**	447.64	620.28	301.04	423.66	y = 0.713x + 3.04	0.99	0.9

Table 3. Values of "r" revealed from simple correlation coefficient analysis of the values on MDA (intra-cellular stress marker) or different redox parameters (any two variables at a time) in the same liver of control, diseased or treated fish *Anabas testudineus*.*p < 0.001; **p < 0.05; and ***p < 0.01. Abbreviations: CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.

Orrelation	Mda	Cat	Sod	
Control	CAT	-0.971***		
	SOD	-0.944***	+0.973***	
	GPx	-0.967***	+0.984***	+0.993***
Diseased	CAT	-0.911***		
	SOD	-0.868**	+0.975***	
	GPx	-0.966***	+0.938***	+0.890***
Melatonin	CAT	-0.920***		
	SOD	-0.964***	+0.901***	
	GPx	-0.977***	+0.936***	+0.963***
Melatonin+ CAT MB(100 mg l^{-1})	-0.902***			
	SOD	-0.952***	+0.889***	
	GPx	-0.936***	+0.926***	+0.986***
Melatonin+ CAT MB(200 mg l^{-1})	-0.973***			
	SOD	-0.928***	+0.975***	
	GPx	-0.969***	+0.955***	+0.908***

Activity of antioxidative enzymes in the liver: Generation of a large number of reactive oxygen species occur during the parasitic infection of fish, causes elevated oxidative stress. CAT, SOD, and GPx form the main enzymatic defense system against the harmful effects of free radicals. In this study, we found a significant decrease in antioxidative enzymes in the diseased condition that support the ill health of fish. But after treatment by MB in combination with melatonin, the level of antioxidative enzymes (CAT, SOD, and GPx)

significantly increased, which promotes good health of the fish (Figure. 6.). Melatonin is a potent free radical scavenger, may decrease the free-radical damage in the liver tissue to activate the major antioxidative enzymes like CAT, SOD, and GPx which metabolize free radicals to reduce oxidative stress in vivo (Tan et al., 1993).

Correlation between MDA and antioxidant enzymes: Correlation coefficient analysis of the data revealed a significant ($p < 0.001$) negative correlation between the hepatic levels of MDA and the activity of CAT, SOD, and GPx in the liver of control, diseased and MB with melatonin-treated fish (Table. 3.). However, a significant positive correlation was found among the values between CAT, SOD, and GPx (Table. 3.). The results of the correlation coefficient analysis fitted with the findings on linear regression analysis (Figure. 7.).

CONCLUSION

This study concludes that MB in combination with melatonin, may minimize the free-radical damage and ultimately lead to improving the fish's health quality. Obtained data are consistent with earlier findings on carp in which direct stimulatory effects of melatonin on the enzymatic and nonenzymatic antioxidants were suggested. Notably, the data presented in this study demonstrate the physiological actions of melatonin and MB in reducing oxidative stress in the liver and accelerating the wound healing in parasite-infected fish. The findings from this study show the therapeutic effect of MB in combination with melatonin to reduce oxidative stress and to cure the parasitic infection of the Hexamitadae family. Our findings might help the aquaculture industry to control the protozoan infection specifically the most mortality causing protozoan parasite of the Hexamitadae family. This may be used as a curing measure to control the infection and reduce mortality of cultured fish but further research is required to make this protocol perfect.

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Conflict of interest: The authors have no conflict of interest to declare.

Data availability statement: The data that support the findings of this study are available from the authors upon reasonable request.

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Influence of Pranic Agriculture Technique on Growth and Yield of Marigold, Tagetes erecta

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ABSTRACT

Agriculture is highly mechanized and crops are grown with excess application of chemical fertilizers and pesticides which are creating environmental pollution and health problems among population. So, adaptation of ancient agriculture techniques which are supportive in achieving sustainable agriculture goals is hightly needed. Pranic agriculture (PA) is one among those ancient farming methods, which utilizes prana or life energy to obtain higher crop growth and yield with no extra inputs. Marigold is one of the most popular, multipurpose annual flowering crops cultivated in Karnataka and different parts of India. The present study was conducted to understand the influence of Pranic energy application on vegetative and reproductive traits of Marigold. Pranic energy was applied by a trained pranic healer to land and seed before sowing and to the crop at the time of growth. Experiment was carried during Kharif 2019 at farmer's field in 0.2 ha area for each treatment. Observations were recorded on different traits and data was analyzed using t-test at probability of < .05. The percent increase in plant spread of pranic treatment over control was significantly higher by 21.01% at 15 DAT, 20.49 % at 30 DAT, 17.05 % at 45 DAT, 16.38% at 60 DAT and 16.88 % at 75 DAT respectively. Number of branches were significantly higher in pranic treatment (12.6) as compared to control (11.1). The number of flowers, flower diameter and yield per plant were significantly higher in pranic treatment (72.1, 5.77cm and 587.6 g) as compared to control (58.26, 4.87cm, 449.7g) respectively. Plant growth and flower yield of marigold was enhanced by the application of pranic agriculture technique. Exact mechanism involved in the improvement of growth and yield needs to be understood. PA would be a supportive farming system in attaining sustainable and eco-friendly agriculture and improve the farmer economic status.

KEY WORDS: FLOWER YIELD, HORMONE, PLANT SPREAD, PRANA.

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INTRODUCTION

Marigold (*Tagetes erecta*. L) is a traditional, multipurpose flower cultivated throughout the world. In India, it accounts for more than half of the loose flower production. Marigold belongs to *Asteraceae* family and is grown all around the year. It is used for religious/ spiritual functions and is available in many colours like yellow and orange. Marigold flowers are used as natural

food flavour and colourant. It also contains carotenoid, which acts as an antioxidant (Narsude et al., 2010). Marigold flower extract is an ingredient in preparing ulcer curing ointment and dietary supplement in the poultry industry to enhance the chicken skin colour and egg yolk pigmentation.

Insect pest damage is one of the major threats in agriculture production (Shivakumar and Srinivasa, 2017). Different types of pesticides are applied against this, which ended in build up of resistance to insect and its residues on the crop is entering the food chain, and also causing pollution. Marigold is one of the important trap crop grown in between main crops like tomato, potato, gourds, grams and other foliage crops to control the attack of nematodes and fruit borer (Srinivasan et al., 1994). Being a multi-utility crop, there is a need to improve the flower yield in marigold.

According to Master Choa Kok Sui (Sui, 2015), Pranic agriculture is a science and art of energy treatment to the plant with "Prana". Prana refers to the energy field called bioplasmic energy surrounding living organisms like plant, animal and human. Prana is a life force or life energy which is subtle and can be observed with Kirlian's photography and is called "Aura" (Kirlian,1949). Pranic energy treatment is given externally to the plants by a pranic healer, utilising the natural prana available in sun, air, ground and water (Sui, 2015). Several studies have been conducted in crops like tomato, cucumber, pole beans, European cucumber, Drumstick, Brinjal and Papaya with pranic energy application and unparallel results were obtained in seedling vigour, plant growth, yield, antioxidant content and shelf life qualities (Jois et al., 2016; Jois et al., 2017; Yathindra et al., 2017a; Yathindra et al., 2017b; Prasad and Jois, 2019; Jois et al., 2019; Prasad and Jois, 2020).

By practising pranic agriculture, farmers can obtain additional higher yield and nutritious crop along with conventional inputs. As the pranic agriculture technique is simple to learn and practice, the farmer can get trained and practise in their field and obtain the benefits (Prasad and Jois, 2020). With these promising crop improvement results and technology access to the farmer, a study was conducted to investigate the effect of pranic energy application on plant growth and flower yield of the marigold crop. It was a first attempted pranic agriculture study on a flower crop.

MATERIAL AND METHODS

For plant material, seeds of Marigold (*Tagetes erecta* L.) Tennis ball variety was used in this study. Pranic agriculture experiment was conducted during Kharif 2019 under field condition. Each treatment was carried out in 0.2 ha land area on red loamy soil. Timely required agronomic cultural practices like weeding, irrigation and earthing up were carried out as per the package of practices. Seeds and the land were divided into two, namely control and treated (Pranic).

During Treatment, Pranic energy is applied by a trained pranic healer. Pranic energy was given for twice a week for two weeks with fifteen minutes each time to the seeds, cocopeat and land of pranic group. Cocopeat, seeds and land which did not receive any treatment were considered as control. Seeds were sown into tray pots containing cocopeat and watered regularly and grown for 20 days till the plant reaches 4 to 5 cm. Vegetative and reproductive parameters were recorded. In each treatment 25 observations were recorded at respective time intervals.

While observing the vegetative parameters, plant height (cm) and plant spread in east-west and north-south (cm^2) directions were recorded at every fortnight interval at 15, 30, 45, 60 and 75 days after transplantation (DAT) using a measuring scale. Other growth parameters like number of branches, leaf area (cm2) and root length (cm) at the grand growth stage (45 DAT) were included. Total Chlorophyll content of leaves was estimated using Dimethyl Sulphoxide (DMSO) in young leaves at 45 DAT (Shoaf and Lium,1976). Fresh leaf tissue of 100 mg was cut into small pieces and incubated in 7 ml of DMSO at 65 ^{o}C for 30 minutes. At the end of the incubation period, the supernatant was taken and made to 10 ml with DMSO, and the absorbance of the extract was read at 652 nm using DMSO as a blank. The total chlorophyll content was calculated by using the following formula and expressed on a fresh weight basis (mg g^{-1}fr.wt).

$$\text{Total chlorophyll} = 27.8\,(A_{652}) \times \frac{V}{1000 \times W \times a}$$

Where, a = Absorbance at 652 nm wavelength, V = Final volume of the chlorophyll extract (ml). W= Weight of leaf sample (g)

While observing the reproductive parameters, time of flowering and flower yield were two important reproductive parameters in marigold. Days taken for bud initiation, first flowering, 50% flowering and first harvest were recorded in both pranic and control treatments. Flower characteristics like the number of flowers per plant and flower weight (g) were recorded. Flower yield from each picking was cumulated and expressed as yield per plant (g) and yield per acre (t). Statistical analysis by t-test was carried out for vegetative and reproductive parameters using Microsoft Excel and SPSS-21.0 and the level of significance was expressed at 5% ($p < .05$). Percent difference between mean values of traits of pranic treatment over its control treatment is calculated and presented as an increase percentage.

RESULTS AND DISCUSSION

Vegetative parameters: Plant height and spread measurements were recorded at 15, 30, 45, 60 and 75 days after transplanting (DAT). Plant height and spread east-west or north-south directions are inter-related and dependent parameters (Table 1).

The difference in Plant height was non-significant among pranic and control treatments initially at 15 DAT, but interestingly as the days progress the plant height increased at a higher rate in pranic treatment. The percent change in plant height was ranging from 0.38 to 3.26 at 15 to 75 DAT. Percent increase in pranic treatment plant height was high in control initially at 15 DAT (0.38%), then increased in pranic by 1.82%, 3.0%, 3.81% at 30, 45 and 60 DAT respectively as compared to control. At 75 DAT the growth in both the treatments reduced by 3.36% as the crop growth shifted to the reproductive phase. In a similar study, Pranic treated tomato plants showed higher plant height (114.24cm) and stem diameter (16.07mm) as compared to control (92.95 cm and 14.02 mm). Pranic energy applied to tomato plant showed an increase of 18.5% and 12% in plant height and plant spread against its untreated group (Jois et al., 2016).

Table 1. Effect of pranic treatment on plant height(cm) and plant spread (cm^2).

	Treatments	Pranic		Control		Change (%)	t-stat	Critical value
		Mean	S. D	Mean	S. D			
Plant height	15 DAT	28.66	7.34	28.77	6.68	-0.38	-0.05	2
	30 DAT	35.23	7.39	34.6	6.65	1.82	0.34	2
	45 DAT	40.02	7.55	38.86	6.77	3.00	0.62	2
	60 DAT	43.00	7.64	41.42	6.80	3.81	0.84	2
	75 DAT	45.16	8.10	43.69	6.92	3.36	0.75	2
Plant spread (East- West)	15 DAT	26.96	3.01	22.28	3.58	21.01	5.48*	2
	30 DAT	30.99	3.01	25.72	3.60	20.49	6.14*	2
	45 DAT	38.59	3.09	32.97	3.44	17.05	6.65*	2
	60 DAT	42.34	3.11	36.38	3.38	16.38	7.09*	2
	75 DAT	44.24	3.22	37.85	3.48	16.88	7.37*	2
Plant spread (North- South)	15 DAT	31.01	4.34	23.37	5.07	32.69	6.27*	2
	30 DAT	35.05	4.32	26.81	5.10	30.73	6.75*	2
	45 DAT	42.64	4.24	34.06	4.62	25.19	7.49*	2
	60 DAT	46.4	4.29	37.47	4.42	23.83	7.93*	2
	75 DAT	48.29	4.32	38.94	4.64	24.01	8.07*	2
* -Significant at p< .05								

Plant spread was recorded in east–west and north–south directions during 15, 30, 45, 60 and 75 DAT. There was a significant variation between pranic and control treatments for plant spread in both directions. The mean value of plant spread in east-west direction was highest in pranic (26.96, 30.99, 38.59, 42.34 and 44.24) as compared to control (22.28, 25.72, 32.97, 36.38 and 37.85) at 15, 30, 45, 60 and 75 DAT respectively. The percent increase in plant spread (east-west) of pranic treatment over control was significantly higher by 21.01% at 15 DAT, 20.49 % at 30 DAT, 17.05 % at 45 DAT, 16.38% at 60 DAT and 16.88 % at 75 DAT respectively. The mean value of plant spread in north-south direction was highest in pranic treatment (31.01, 35.05, 42.64, 46.40 and 48.29) as compared to control (23.37, 26.81, 34.06, 37.47 and 38.94) at 15, 30, 45, 60 and 75 DAT respectively.

The percent increase in plant spread (north-south) in pranic treatment over control was significantly higher by 32.69 at 15 DAT, 30.73 at 30 DAT, 25.19 at 45 DAT, 23.83 at 60 DAT and 24.01 at 75 DAT respectively. The total number of branches are significantly higher in pranic treatment (12.6) compared to control (11.06) (Table 2). Even though leaf area and root length were non- significant between treatments, but the highest was observed in pranic treatment (108.15 and 14.02) as compared to control (103.5 and 11.26) respectively (Table 2). Total chlorophyll content was also on par in both the treatments but numerically highest in pranic treatment (3.75) as compared to control (3.42) (Table 2).

In the present study, plant height variation is non-significant between the treatments, but plant spread is significantly higher in pranic treatment. Plant growth and development and its shift from the vegetative phase to reproductive phase depend on endogenously produced plant hormones which are regulated by inherent genetic characters and external environmental influences (Gray,2004). It can be hypothesised that ratio between auxin and cytokinin might have been altered by external pranic energy treatment and have lead to the higher lateral plant spread and a higher number of branches. The well-known theory that, auxin contributes for apical dominance and cytokinin for lateral dominance and the ratio between two hormone plays a major role in branching and bud initiation in flowering plant, is parallel support for the pattern of growth in pranic treated plants (Muller and Leyser, 2011; Kebrom, 2017).

Present study results were in agreement with Bairwa and Mishra (2017), where different doses of auxin and cytokinin have shown a change in plant growth pattern in African marigold. In this study different types of

hormones viz. auxin (NAA at 100,200 and 300 ppm) and cytokinin (BA at 25, 50 and 75 ppm) and (Kinetin at 50,100 and 150 ppm) were applied at different concentrations. Among different treatments NAA @ 300 ppm recorded maximum plant height (77.26 cm), the number of branches (14.53), plant spread (60.80 ×56.86 cm2.The increase in growth and yield might be because NAA enhance cell division and expansion and tissue growth. Similar inhibition was observed in the linear growth of African marigold plant with a higher concentration of NAA (Disha et al., 2014; Kebrom, 2017).

Reproductive parameters: Flowering traits like days to bud initiation, days to first flowering, days to 50% flowering and number of days taken for the first harvest were initiated early in the pranic treated plot as compared to control plot (Table 3).

Table 2. Effect of Pranic treatment on plant morphology and chlorophyll content

Treatments	Pranic		Control		Increase (%)	t-stat	Critical value
	Mean	S.D	Mean	S.D			
Number of branches	12.6	2.39	11.06	2.39	12.22	2.48*	2
Leaf area (cm2)	108.15	10.11	103.5	5.8	4.49	0.79	2.44
Root length (cm)	14.02	3.09	11.26	2.92	24.51	1.45	2.3
Total Chlorophyll content (mg g -1fr.wt)	3.75	0.1	3.42	0.28	9.65	2.06	2.44

* -Significant at p< .05

Days taken for first flower initiation were significantly minimum in pranic treatment (64.1) as compared to control (68.26). Days for bud initiation, 50% flowering and days for first harvest were found minimum in pranic treatment (59.53, 103.5 and 91.25) as compared to control (60.86, 106 and 93.25) respectively.In a similar pranic agriculture study on pole beans (*Phaseolus vulgaris* L.), a reduction in time duration to flowering and flowering to fruit set by 2.2% and 3.2% respectively was noticed in pranic treated groups when compared to control. Mean values of flowering date and flowering to fruit set was lower in pranic treatment (30.4 and 31.2 days) as compared to control (31.1 and 32.2 days). Fruit yield of pranic treatment (0.49 kg) is higher than control (0.44 kg). The probable reason for the improvement was attributed for alterations in the molecular structure of treated cells, affect nucleotide polymerisation, gene expression and enzyme activity (Bai et al., 2000; Yathindra et al., 2017b).

Table 3. Effect of pranic treatment on flowering parameters and flower yield.

Treatments	Pranic		Control		Increase (%)	t-stat	Critical value
	Mean	S.D	Mean	S.D			
Days to bud initiation	59.53	4.89	60.86	5.32	-2.19	-1.01	2
Days to first flowering	64.1	6.58	68.26	7.86	-6.09	-2.22*	2
Days to 50% flowering	103.5	1.29	106	2.58	-2.36	-1.73	2.44
Number of days taken for first harvest	91.25	3.5	93.25	1.71	-2.14	-1.02	2.44
Number of flowers per plant	72.1	9.66	58.26	6.57	23.76	6.84*	2
Flower weight per plant (g)	8.16	0.62	7.69	0.83	6.11	1.74	2.04
Flower diameter (cm)	5.77	0.3	4.87	0.32	18.48	7.84*	2.04
Yield per plant (g)	587.6	83.34	449.7	75.46	30.68	6.72*	2
Yield per acre (t)	6.09	0.81	4.66	0.81	30.69	2.47*	2.44

* -Significant at p< .05

Cultivar Arka Bangara-2 showed minimum days to flower bud initiation (39.54), days to 50 percent flowering (51.7) and longest flower duration (77.29). Flower diameter was highest in cv. Maxima yellow (7.20 cm) followed by cv. Arka Bangara-2 (6.17 cm). The variation was attributed to the prevailing climatic condition of the experimental location. The present results are also in conformity with the findings in marigold (Narsude et al., 2010a). The number of flowers per plant, flower weight, flower diameter was found significantly higher in pranic treatment (72.1, 8.16 and 5.77) as compared to control (58.26, 7.69 and 4.87) respectively (Table 3).

Yield per plant (g) and yield per acre (t) were significantly higher in pranic treatment (587.6 and 6.09) as compared to control (449.7 and 4.66) respectively.

Similarly, Pranic agriculture study in European cucumber showed a significant reduction in time duration to flowering in pranic treatment (54.3 days) as compared to control (59.1 days). Pranic treatment showed higher yield per plant (1.9 kg/plant) as compared to control (1.58 kg/plant). This change accounts for 14 % and 18 % increase in the number of fruits per plant and higher yield in pranic treatment against its control (Yathindra et al., 2017b).

Performance of any crop is decided by its economic yield. The number of marigold flowers per plant and flower weight was found 23.76% and 6.11% higher in the pranic treated plot over and above its control.

Similarly, flower diameter and yield per plant were improved in pranic treatment by 18.48% and 30.68% over its control. Similarly, 30.69% higher yield per acre was found in the pranic plot over its control plot. A similar study on tomato plant treated with pranic energy showed higher flowers per plant (10.14) and yield per plant (117.07 kg) as compared to control (10.36 and 80.58) and this increase in number of flowers and total yield per plant accounts for 31.75% and 31.10% higher than control (Jois et al., 2016).

In another pranic agriculture study on Cucumber also showed similar results to the present study. Plant height, stem diameter and fruit yield of pranic treatment (28.77 inches, 7.45mm and 63.84 kg) as compared to control (14.60 inches, 5.65 mm and 52.85 kg) respectively. The influence of pranic energy probably has improved cellular growth and division and increased ATPase activity and has a positive effect on growth and yield (Jois et al., 2017). In another similar pranic agriculture study in Papaya seedlings, pranic energy treated seeds showed significant ($p < .05$) variation for mean germination days (11.3), shoot length (7.3 cm), number of leaves (7.6), leaf length (3.4cm), leaf diameter (2.9 cm) and seedling vigour index I (2350) and seedling vigour index II (1564) as compared to untreated seeds (14.7, 5.6 cm, 5.1, 2.1 cm, 1.7 cm, 1626 and 624) respectively (Prasad and Jois, 2020).

Pranic energy supplied to the seeds during cell division, growth and differentiation probably resulted in enhanced growth of pranic treated papaya.The flowering time and time of transition from vegetative to reproductive development is affected by environmental conditions as well as hormonal action (Muller and Leyser 2011; Davies et al., 2010. The externally applied pranic energy might have brought changes in the hormonal signalling pathway at the cellular receptor level and lead to the variation in the flowering duration and flower yield (Denay et al., 2017; Campos et al., 2017). In a similar study different types of hormones viz. auxin (NAA at 100,200 and 300 ppm) and cytokinin (BA at 25, 50 and 75 ppm) and (Kinetin at 50,100 and 150 ppm) were applied at different concentrations. Among different treatments NAA @ 300 ppm recorded maximum number of flowers per plant (57.60), the average weight of flowers (12.93g), the average diameter of flowers (9.20 cm) and yield of flowers per plant (744.7 g), flowers per plot (7.36 kg) and flowers per hectare (170.37 q) (Bairwa and Mishra., 2017).

The number of flowers produced per plant is directly related with the number of branches per plant. Higher plant spread and a number of branches in pranic treatment resulted in more photosynthesis due to higher source capacity with enhanced food accumulation. Further higher source capacity might have resulted in better transportation to sink and subsequently a bigger and higher number of flowers per plant. Similar findings have been reported in African marigold with thirteen different nutrient treatments with two biofertilizers (Azotobacter and PSB) combinations. Treatment, T11(PSB+ *Azaobacter*+Full K+FYM+half N and P) recorded maximum plant spread (3420.1 and 3502.6 cm), number of branches per plant (13.59 and 15.69), number of days for bud initiation(48.20 and 45.12 days), first flower opening (9.30 and 11.65 days, marked increase in flowering span (41.39 and 45.79), flower diameter(7.10cm 8.54cm), number of flowers per plant (28.93 and 29.44 cm) compared to other treatments at year the 2004 and 2005 respectively (Bairwa and Mishra., 2017).

Combination of biofertilizers and organic manure has reduced to dosage of organic fertilizer by 50% was advantageous (Kumar et al., 2019). Similar improvements in Marigold flower diameter (7.39 cm), flower weight (7.43 g), number of flowers per plant (52.37), flower yield per plant (388.33g) and yield per hectare (14.38t) were recorded with the application of integrated nutrient combination (70%RDF+Vermicompost+Azotob acter+ Azospirillum + PSB) followed by other nutrient combinations and control (Mittal, et al., 2010). The results were attributed to the constant and optimal supply of nutrients throughout the growth period influenced better growth.

In a study of Okra, seeds were treated with a magnetic field of 99 mT for 3 min and 99 mT for 11 min exposure before sowing. Treatment of 99 mT for 11 min showed significant variation in germination percentage (71), number of flowers per plant (21.75), plant height at maturity (102), number of pod per plant (16), pod mass per plant(692), and number of seeds per plant (133) as compared to control (53, 7.25, 73, 10, 350 and 59) respectively. The effect of treatment is not only dependent on the magnetic field but also the duration of exposure (Naz et al., 2012; Kumar et al., 2019).

CONCLUSION

Pranic energy application on marigold has resulted in higher plant spread and a higher number of flowers per plant ultimately leading to higher yield per plant. Pranic agriculture can be adopted easily by the farmer himself in his field by learning the pranic healing

technique without any additional input cost. Further studies are in progress to understand the mechanism of pranic energy influence on the plant at the cellular and whole plant level. PA is an easy technique to learn by farmers and can apply to their crops to fetch higher yield and improve their economic status. PA can become one of the supportive farming system in coordination with conventional farming methods to achieve global agriculture sustainability.

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The Influence of Social Media on Developing English Reading Skills of Saudi Universities Students

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ABSTRACT

Social media has definitely become an integral part of our lives especially among university students. This technology has a wide impressive effect on how people learn and communicate with each other. However, the application of social media to learning especially for English department Saudi university students, is not fully explored. Therefore, the current study aims at mapping whether social media affects students' reading competence positively or negatively. The study also seeks to find out if there are any significant differences in the students' competence in reading as a result of using social media with NBU students due to gender, social media, reading skills and geographical distribution. The current study used quantitative methods using a survey instrument to gather descriptive data regarding the perceptions of (n=900) randomly chosen Saudi university students. Questionnaires with Likert scale questions were used in the study. The questionnaire was divided into two main parts. The first part began with two general questions about the number of years by which social media was used to perform general tasks and to perform specific English tasks. The second part was concerned with attitudes towards using social media, impact that social media has on reading competence. The data was analyzed using the Statistical Package for Social Sciences (SPSS) to obtain accurate results. Analysis of the collected data showed that the majority of the participants believed strongly in the pedagogical values and benefits of using social media as an ELT tool in their classrooms especially in improving reading skills. The findings also indicate that social networking websites and applications (e.g.Facebook, Youtube , Twitter, Skype) have a positive impact on learning English as a foreign language.

KEY WORDS: SOCIAL MEDIA, ENGLISH READING SKILLS, UNIVERSITY STUDENTS.

INTRODUCTION

Nowadays, the internet has become one of the most powerful avenues for disseminating information. The everyday use of the internet has played a major role in the improvement of students' English language and has brought about significant changes in the way students find, manage and use information. The internet has been designed to meet an array of quite different purposes, some of which are certainly educational (AlQahtani,2018). The social media include Facebook, Instagram, Twitter, Snapchat, and Whatsapp, etc. It facilitates learners easy to access at any time at any place. The rapid use of social media applications has become an order of the day for numerous purposes. The new users and apps emerge every day across the region enable one and all to express personal views, ideas, opinions, share research/ educational projects, blogs writing, social networking sites, and cyber virtual space.

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The social media has a few well-established applications of social media. These apps are gaining a lot of interest among Saudi learners in academics alike, which exhibits interest to fetch constructive results using these apps (Sharma,2019). Reading has been a great source of information and knowledge at all the times and in all ages. Reading is one of the oldest cultures for human progress in society. The ability to read and write is highly valued and essential for social, cultural and economic advancement.

The regular and systematic reading develops the cognitive ability and improves communication skills. Reading habit is the most fundamental skill that is necessary for the success not only for academic purpose but for all walks of life . As Many studies (Junco et al., 2011; Madge et al., 2009; Olutola et al., 2016) reported that positive use of social media improved the reading habits and academic performance of students. On the other hand, Shabir et al. (2014) and Lubis et al. (2012) found that there was no significant relationship between the use of social media and academic performance (Rafiq etal.,2019). Therefore, this paper aims to investigate the ways in which social media can improve reading skills among Saudi university students.

Study questions: This paper seeks to answer the following questions:

1. What is the effect of using social media on Northern Border University students' reading proficiency?
2. Does gender affect English language learning and is reading improved by the use of social media?
3. Does the geographical location (distribution) of NBU among other Saudi universities make a difference in improving reading via social media?
4. What are the constraints that students face when using social media to improve reading proficiency?

Objective of the study: The present study aims at Mapping whether social media affects students' reading competence positively or negatively,addition to find out if there are any significant differences in the students' competence in reading as a result of using social media with Northern Border University students due to gender, social media, reading skills and geographical distribution / location.

Research design: This study used a questionnaire to collect data. The study used Likert scale statements where each statement had five Likert-type items from which participants could choose.. The questionnaire was divided into two main parts. The first part started with two general questions about the number of years in which social media had been used in general and the number of years in which social media had been used to perform some tasks in English. The second part was comprised of three main components. The first component was concerned with aattitudes towards using social media as a tool for reading in the English language. The second component addressed the specific impact that social media has on reading competence. The third component focused on the effect of social media on reading proficiency in respect to gender and location. The data was analyzed using the Statistical Package for Social Sciences (SPSS) to obtain precise results.

Participants: The sample for the study were randomly selected by using a systematic sampling method consisted of nine hundred students who studied in the English department in the first year in the first semester of the 2019 academic year at Northern Border University in Saudi Arabia.

Definition of terms: Reading is the process of looking at a series of written symbols and deducing their meaning. It is a receptive skill through which we receive information. It requires a level of skill in speaking, so that the read words can be pronounced. There are four types or skills in reading as follows:

- Intensive
- Skimming
- Scanning
- Extensive

Reading is defined as a cognitive process that involves decoding symbols to arrive at meaning. Reading is an active process of constructing the meaning of words. Reading with a purpose helps the reader to direct information towards a goal and focuses their attention. Although the reasons for reading may vary, the main purpose of reading is to understand the text. Reading is a thinking process. It allows the reader to use what he or she may already know, also called prior knowledge. During this process of acquiring information, readers use strategies to understand what they are reading, use themes to organize ideas, and use textual clues to find the meanings of new words. (https:llstudy.com). According to Crutis and Kruidenier (2005) Reading comprehension is the process of constructing meaning from what is read. To comprehend, a reader must decode words and associate them with their meanings.

Social media: Social media consists of online communication channels dedicated to community based input, interaction, content sharing and collaboration. Websites and applications dedicated to forums, micro blogging, social networking, social curation and wikis are among the different types of social media (https:llwhatis.techtarget.com).

Importance of the study: Reading activity is the foundation of learning new knowledge and gaining new skills, and comprehension is at the heart of reading (Chen, Teng, Lee, and Kinshuck, 2011). Research on reading found that skilled readers focus on reading as a process of gaining meaning, as opposed to readers who focus on reading as a decoding process, which makes them unsuccessful or unskilled readers (Baker and Brown 1984). Decoding is turning the written word into its spoken and known equivalent while comprehension is the construction of meaning (Nation, 2008). When a

reader uses too much of his/ her cognitive resources to decode, insufficient cognitive resources will be available to understand and make sense of what is read (Rasinski and Hoffman, 2003). Roberts (2011) claimed that too much time and energy spent decoding text leaves little time and energy for constructing meaning. Positively speaking, Rasinski (2012) added that with practice the lower level processes can become automatic which means a reader no longer needs to apply conscious attention to decoding and thus can become a fluent reader. In the context of reading, such technology provides a way to communicate actively, allowing students to have discussions both inside and outside the classroom. Learning is considered a social process that can be enhanced through dialogue.

Almarwany (2017) showed that in recent years social media such as Facebook, Twitter and other forms are considered useful tools for social interaction, communication and sharing information. Most scholars state that students use social media to support their emotions, to express themselves freely, and enhance their social life. Many researchers have shown the positive effects of using social media in learning English as a foreign language. When students are free to express themselves at a time and place that suits them, they can express themselves using language fluently, as a lot of students may be shy when using English as a foreign language. So with social media, they can read, write and speak without being afraid of making mistakes.

Looi (2011) stated that from a social constructivist point of view, reading is considered to be a social practice. The rise of web 2.0 tools such as blogs, wikis, and social networking sites, has provided a wide range of opportunities to create active interactions among learners inside and outside the school. In the past, students were merely receptive learners of knowledge, but nowadays, students are encouraged to be active members in a learning society. They are active constructors of their own learning environment and no longer learn in isolation. This study suggests that social media can be of great use. For example, Edmodo can be useful in improving reading comprehension among adult learners of English as a foreign language. This is due to learners' positive perceptions and attitudes, the likelihood of improved performance, and opportunities for participation in the learning community. It also showed the necessity of integrating social media in teaching and learning. This can greatly enhance the learning of reading and writing in an interesting way.

Manca and Ranieri's study (2016) aimed at identifying the uses of social media in university teaching practices. The study conducted a survey in which participants were asked to identify rate of use, motivations, teaching practices and difficulties related to the use of a number of social network sites (Twitter, Facebook), professional and academic networking services (LinkedIn, ResearchGate and Academia.edu), tools for writing and comment (blogs, wikis) and to archive and retrieve content material for lectures and group work (podcasts, YouTube and Vimeo, SlideShare). Data analysis was used to test those socio-demographic variables that most affected frequency of use, and the relationships between motivations, ways of use, obstacles to use and scientific discipline. The results showed that social media use is still rather limited and restricted in scope and that academics are not greatly motivated to integrate these devices into their practices. There are several reasons for this, such as cultural resistance, pedagogical issues and/or institutional constraints. Overall, the results stress ambivalent attitudes towards the benefits and challenges of social media in the context of higher education with obstacles prevailing over advantages.

Narayan and Sunath's study (2016) examined the effect of media, especially print and social media, on teaching English. With the advent of computers there is more emphasis given to American English and a range of spelling is accepted. The importance of spoken style, grammar and syntax is slowly gaining credence but the convenience of auto spell check has affected the ability of students to spell correctly while some students have become totally dependent on the auto-editing mode. The arrival of social media such as Facebook and Whatsapp has resulted in the overuse of shortened expressions. However, knowledge of these abbreviations seems to be the need of the hour and people who cannot master the art appear to be out of place. It is exactly here that English teachers can step in and help strike a balance between the younger generation and the older generation who are less proficient in social media.

Allam and Elyas's study (2016) is based on quantitative methods using a survey instrument to gather descriptive data regarding the perceptions of 75 randomly chosen English as a Foreign Language (EFL) teachers at two Saudi tertiary institutions. The study used 14 Likert scale statements where each statement had five Likert-type items for the participants to choose from. Analysis of the collected data showed that the majority of the participants believed strongly in the pedagogical values and benefits of using social media as an ELT tool in their classrooms. However, the majority expressed reservations with regard to the extent to which social media should be freely used in the EFL classroom where they recognize it as having a dual effect. The study recommended more research studies in this area so as to closely understand how experienced EFL teachers utilize social media in their classes in order to develop best practices for implementing social media in teaching and learning in EFL in the Saudi context.

Sitthirak's study (2013) investigated how social media has influenced teaching and learning English at present. Social media has been incorporated into an informal education system for decades; i.e. teleconferencing and distance learning before it developed into a more sophisticated system. However, there is still controversy over using new social media such as Facebook or WebChat with conflicting discussions among multiple studies. Ultimately the roles of teachers and learners must be reconsidered along with their affective and attitudinal

effects on social media when used for educational purposes, since it will be or is now inescapably integrated into our daily lives. Alsulami's study (2016) investigated how technology affected learning English as a foreign language among female EFL students at Effatt College.

Questionnaires designed with Likert scale questions were used in the study that was divided into two parts. The first part started with two general questions about age and educational level. The second part included four specific questions about technological tools that could enhance learning the English language. The sample consisted of 36 participants. The data was analyzed using the Statistical Package for Social Sciences (SPSS) to obtain precise results. The findings clearly indicated that computer software, social networking websites, online videos, audio tools (i.e., YouTube, Skype, MP3 players), and smart phone and tablet apps have a positive impact on learning English as a foreign language. Thus, technological tools can clearly be effective in improving the students' language and communication skills.

Monica and Anamaria's study (2014) aimed at investigating the impact of computers and social media on improving students' knowledge of English language especially vocabulary acquisition (focusing on Facebook) with intermediate and upper intermediate first and second year ELT students in Economics at the Faculty of Economic Sciences, University of Oradea. The study was conducted with 127 students of the Faculty of Economic Sciences, University of Oradea, 1st and 2nd year students following specializations in International Business, Management, Marketing, Finances who were studying in the academic year 2013-2014. The development of each group was measured and clearly demonstrated a more significant improvement in vocabulary knowledge among the group exposed to Facebook. Such an innovative study is rare as there are few studies exploring the value Facebook can add to learning in Romania. The results of the study did not support the assumption that the experimental group would outperform the control group, as the differences between the two groups were not particularly significant. However, there was an improvement in both of the groups from pre-test to post-test scores.

Alfahadi's (2017) study examined how social media sites can develop English language skills among Tabuk University students and to determine the most commonly used social media by the learners in order to practice English language skills. The researcher concluded that social media sites can be integrated in the EFL syllabi as teaching and learning aids because they contain a broad combination of sound, text and videos where students can express their viewpoints and receive direct remedial feedback. The study also found that You Tube is the most common social media site used by students at Tabuk University to practice English Language.

Suswati and Saleh's study (2019) investigated social media to assess EFL students at the State University of Medan (UNIMED) in their second semester English educational program. This research concentrated on how social media influences students' writing skills. The problems included how it influenced their ability to develop their ideas in writing; develop reading and writing materials; and change students' opinions on social media in writing class. The questionnaire was designed to gather the students' thoughts on social media matters, their intention to use social media for study, particularly in reading and writing topics and the influence of social media on their ability to develop an idea in writing class.

The instruments used in this research were social media such as Facebook, WhatsApp and Instagram as texts in reading and writing class. The sample consisted of 80 students from two classes in the English educational program. Data was analyzed using the Research & Development Method (R&D) and the Linkert Scale to ascertain the percentage of students' perception. The findings of this paper indicated that students enjoy and engage in writing class more when using social media for their assessments, as well as using social media to develop ideas and be more creative in writing skills.

RESULTS AND DISCUSSION

Data Analysis: This section presents the results of the study. To analyse data, the SPSS program was conducted as shown in the tables and diagrams below to process the data yield including chi-squared analyses.

The First Part:

Table. 1 Frequency and percent of male and female respondents

	Frequency	Percent
Male	120	50.0
Female	120	50.0
Total	240	100.0

Table (1) shows that both males and females responded equally to the questionnaire. This indicates that they were both interested in sharing their experience using social media in learning.

Table 2. what is the social media most used by you

	Frequency	Percent
You Tube	90	37.5
Instagram	20	8.3
Twitter	10	4.2
Snap chat	120	50.0
Total	240	100.0

Table (2) above shows that the social media most commonly used in the study area are: Snap chat 50%, You Tube 37.5%, Instagram 8.3% and Twitter 4.2%.

Table 3. Years of using social media

	Frequency	Percent
Less than 3 years	30	12.5
Valid 3-5 years	172	71.7
more than 7 years	38	15.8
Total	240	100.0

Table (3) shows that 71.7% of the students used social media for 3-5 years.

Table 4. Years of using social media to perform some tasks in English

	Frequency	Percent
Less than 3 years	138	57.5
Valid 3-5 years	52	21.7
more than 7 years	50	20.8
Total	240	100.0

Table (4) shows that 57.5% of respondents used social media to perform tasks in English for less than 3 years.

Table 6. Attitudes towards using social media as a tool for reading in the English language

Questions	Response	Observed	Expected N	Residual	Df	Chi-value	sig
You can read activities in English through utilizing social media	Strongly agree	36	48.0	-12.0	4	57.833	0.00
	Agree	86	48.0	38.0			
	Neutral	64	48.0	16.0			
	Disagree	32	48.0	-16.0			
	Strongly disagree	22	48.0	-26.0			
You can understand the linguistic content of social media texts	Strongly agree	14	48.0	-34.0	4	146,833	0.00
	Agree	114	48.0	66.0			
	Neutral	64	48.0	16.0			
	Disagree	32	48.0	-16.0			
	Strongly disagree	16	48.0	-32.0			
you can access the links eqources easily	Strongly agree	124	80.0	44.0	2	80.400	0.00
	Agree	100	80.0	20.0			
	Neutral	16	80.0	-64.0			
	Disagree	0	0	0			
	Strongly disagree	0	0	0			
You read replies and assignment on the social media easily	Strongly agree	106	48.0	58.0	4	129.333	0.00
	Agree	68	48.0	20.0			
	Neutral	38	48.0	-10.0			
	Disagree	10	48.0	-38.0			
	Strongly disagree	18	48.0	-30.0			
You can read more if the social media is used as a supplementary tool in English courses	Strongly agree	44	48.0	-4.0-	4	114.667	0.00
	Agree	110	48.0	62.0			
	Neutral	46	48.0	-2.0			
	Disagree	26	48.0	-22.0			
	Strongly disagree	14	48.0	-34.0			
Questions	Response	Observed	Expected N	Residual	df	Chi-value	sig
You like to read collaboratively Through social media	Strongly agree	116	48.0	68.0	4	140.667	0.00
	Agree	56	48.0	8.0			
	Neutral	32	48.0	-16.0-			
	Disagree	20	48.0	-28.0-			
	Strongly disagree	16	48.0	-32.0-			
You are more motivated to read when doing reading activities on social media	Strongly agree	132	80.0	52.0	2	54.300	0.00
	Agree	66	80.0	-14.0			
	Neutral	42	80.0	-38.0			
	Disagree	0	0	0			
	Strongly disagree	0	0	0			
You prefer to read in English by using social media	Strongly agree	60	48.0	12.0	4	136,83	0.00
	Agree	112	48.0	64.0			
	Neutral	38	48.0	-10.0			
	Disagree	20	48.0	-28.0			
	Strongly disagree	10	48.0	-38.0			

The Second Part: This part consists of the questionnaire items, which are classified into three components

Table (7) shows that most of the responses tend to agree with the items. The following information is evident: 86 of the sample agreed that they can read activities in English through utilizing social media. 114 of them agreed that they can understand the linguistic content of social media texts. In addition, 124 of the sample strongly agreed that they can access the resource links easily. Also, 106 of the respondents strongly agreed that they understood replies and assignments on social media easily. Of the respondents 110 read more if social media is used as a supplementary tool in English courses. It was agreed by 116 agreed that they liked to read collaboratively through social media while 123 respondents strongly agreed that they are more motivated to read when doing reading activities on social media and 112 agreed with a high level of statistical significance (sig less than 0.05) that they preferred to read in English by using social media.

Table 7.

Questions	Response	Observed	Expected N	Residual	df	Chi-value	sig
You...work better in groups through social media	Strongly agree	74	60.0	14.0	3	93.200	0.00
	Agree	114	60.0	54.0			
	Neutral	38	60.0	-22.0-			
	Disagree	14	60.0	-46.0-			
	Strongly disagree	0	0	0			
Social media stimulates your interest in reading in English	Strongly agree	110	110	62.0	4	159.000	0.00
	Agree	78	78	30.0			
	Neutral	24	24	-24.0-			
	Disagree	14	14	-34.0-			
	Strongly disagree	14	14	-34.0-			
Social media encourages you to contribute to your reading skill	Strongly agree	60	48.0	12.0	4	98.667	0.00
	Agree	100	48.0	52.0			
	Neutral	44	48.0	-4.0-			
	Disagree	24	48.0	-24.0-			
	Strongly disagree	12	48.0	-36.0-			
Social media increases your productivity in reading	Strongly agree	14	48.0	-34.0-	4	139.833	0.00
	Agree	118	48.0	70.0			
	Neutral	48	48.0	0			
	Disagree	28	48.0	-20			
	Strongly disagree	32	48.0	-16.0-			
Social media connects you with your professors at home	Strongly agree	14	48.0	-34.0-	4	277.1905	0.00
	Agree	62	48.0	14			
	Neutral	130	48.0	82.0			
	Disagree	20	48.0	-28.0-			
	Strongly disagree	14	48.0	-34.0-			
Social media has a degative effect on your proper syllables	Strongly agree	54	48.0	6.0	4	169.833	0.00
	Agree	4	48.0	-44.0-			
	Neutral	10	48.0	-38.0-			
	Disagree	34	48.0	-14.0-			
	Strongly disagree	108	48.0	60.0			
Questions	Response	Observed	Expected N	Residual	df	Chi-value	sig
Social media enables you to understand and realize reading easily	Strongly agree	54	48.0	6.0	4	169.833	0.00
	Agree	124	48.0	76.0			
	Neutral	28	48.0	-20.0-			
	Disagree	20	48.0	-28.0-			
	Strongly disagree	14	48.0	-34.0-			
Social media can motivate you into more active and interactive reading	Strongly agree	118	48.0	70.0	4	130,333	0,00
	Agree	26	48.0	-22			
	Neutral	34	48.0	-14			
	Disagree	38	48.0	-10.0-			
	Strongly disagree	24	48.0	-24.0-			
Social media enriches your knowledge to read	Strongly agree	32	48.0	-16.0-	4	160.833	0.00
	Agree	120	48.0	72.0			
	Neutral	58	48.0	10.0			
	Disagree	16	48.0	-32.0-			
	Strongly disagree	14	48.0	-34.0-			
Social media improves your ability in grammar	Strongly agree	22	48.0	-26.0-	4	116.667	0.00
	Agree	114	48.0	66.0			
	Neutral	30	48.0	-18.0-			
	Disagree	38	48.0	-10.0-			
	Strongly disagree	36	48.0	-12.0-			

Table (7) shows that there is a very substantial trend towards agreement on the items, with a high level of statistical significance. Among the respondents 114 agreed that they work better in groups through social media and 110 strongly agreed that social media stimulates their interest in reading in English. One hundred of the respondents agreed that social media encouraged them to contribute to their reading skills.1 One hundred and eighteen respondents agreed that social media increased their productivity in reading. Also 130 agreed that social media connected them with their

professors at home. One hundred and thirty respondents had neutral responses to social media as a means to connect with their professors at home while 108 strongly disagreed that social media had a negative effect on their proper syllables. One hundred and twenty-four agreed that social media enabled them to understand and realize reading easily. One hundred and eighteen respondents strongly agreed that social media could motivate them into more active and interactive reading, and 120 agreed that social media enriched their reading knowledge. Also social media improved their ability in grammar, with a high level of statistical significance (sig less than 0.05) which means social media had a positive impact on reading competence in the study area.

Table 8. The effect of social media on the reading proficiency

Questions	Response	Observed	Expected N	Residual	df	Chi-square	sig
Your reading proficiency is inadequate as a result of the absence of internet facilities.	Strongly agree	42	48.0	-6.0-	4	124.333*	0.00
	Agree	106	48.0	58.0			
	Neutral	64	48.0	16.0			
	Disagree	14	48.0	-34.0-			
	Strongly disagree	14	48.0	-34.0-			
The proficiency of female students in English reading is better than their male counterparts.	Strongly agree	0	0	0	4	190.800	0.00
	Agree	20	60.0	-40.0-			
	Neutral	30	60.0	-30.0-			
	Disagree	152	60.0	92.0			
	Strongly disagree	38	60.0	-22.0-			
Male students have ran ive social media more than females	Strongly agree	66	48.0	18.0	2	255.833*	0.00
	Agree	120	48.0	72.0			
	Neutral	30	48.0	-18.0-			
	Disagree	12	48.0	-36.0-			
	Strongly disagree	12	48.0	-36.0-			
The accessibility of internet service centers and services are restricted.	Strongly agree	60	48.0	12.0	4	255.833	0.00
	Agree	140	48.0	92.0			
	Neutral	20	48.0	-28.0-			
	Disagree	10	48.0	-38.0-			
	Strongly disagree	10	48.0	-38.0-			
The social culture of my Area has an effect on my usage of social m e d i a	Strongly agree	80	48.0	4.0-	4	164.16*	0.00
	Agree	110	48.0	62.0			
	Neutral	20	48.0	-2.0			
	Disagree	20	48.0	-22.0			
	Strongly disagree	10	48.0	32.0			
Questions	Response	Observed	Expected N	Residual	df	Chi-square	sig
The inadequate reading can be moderately solved by enabling more use of social media	Strongly agree	50	48.0	2.0	4	189.16*	0.00
	Agree	130	48.0	82.0			
	Neutral	20	48.0	-28.0-			
	Disagree	20	48.0	-28.0-			
	Strongly disagree	20	48.0	-28.0-			
Social media makes you more communicative with each other regardless of the geographical distance.	Strongly agree	40	48.0	-8.0-	4	471.458	0.00
	Agree	180	48.0	132.0			
	Neutral	10	48.0	-38.0-			
	Disagree	5	48.0	-43.0-			
	Strongly disagree	5	48.0	-43.0-			
More use of social media makes your reading proficiency better	Strongly agree	120	48.0	72.0	4	271.458	0.00
	Agree	100	48.0	52.0			
	Neutral	10	48.0	-38.0-			
	Disagree	5	48.0	-43.0-			
	Strongly disagree	5	48.0	-43.0-			

Table (8) shows the effect of social media on the reading proficiency with respect to gender and location. It seems that most of the responses tended to agree to items with the following information being evident: 106 of the sample agreed that their reading proficiency was inadequate as a result of the absence of internet facilities, but 152 disagreed that female students were more proficient in English reading than their male counterparts. In addition, 120 agreed that male students could use social media more than females. Of the sample, 140 agreed that access to Internet service centers and services was restricted. One hundred and ten of the sample agreed that the social culture of their area had an effect on their usage of social media. Within the sample 110-130 agreed that inadequate reading can be moderately solved by enabling more use of social media, while 180 of the sample agreed that social media made them more communicative with each other regardless of geographical distance. Also 120 of the sample strongly agreed that greater use of social media improved reading proficiency with a high level of statistical significance (sig less than 0.05).

From the above analysis and discussion, the results revealed that the most university students use social media in their area of study (You Tube, Snap chat, Instagram and Twitter), the most students had used social media for 3-5 years but they had used it to do some tasks using English language for less than 3 years, most of the students in the study area can read activities in English by utilizing social media. They can also understand the linguistic content of social media texts. In addition they are able to access the links resources, Most students in the study read more if social media was used as a supplementary tool in English courses, Most students in the study enjoyed reading collaboratively through social media, Respondents confirmed that they are more motivated to read when doing reading activities on social media and they preferred to use social media to read in English, Social media encouraged the students in the area of study to improve their reading skills and also increased the amount they read, social media had a positive effect on their correct use of syllables, and social media enriched the reading knowledge of the students in the area of the study knowledge and also improved their ability in grammar.

Also , the results indicated that social media had a positive impact on reading competence in the study area with a high level of statistical significance (sig less than 0.05), university students in the study area considered that their reading proficiency was inadequate as a result of the absence of Internet facilities, respondents emphasized that the proficiency of female students in reading English was not superior to their male counterparts, university students in the study area considered that male students used social media more than females, the social culture of the study area had no effect on students' usage of social media, inadequate reading can be somewhat improved by enabling more use of social media, social media enables students to be more communicative with each other regardless of geographical distance, and greater use of social media improved reading proficiency of university students in the study area.

CONCLUSION

The study concluded that social media has a positive influence on learning reading English as a foreign language in Saudi universities. To achieve this objective, those who teach and those who learn English as a foreign language should be well trained in new technology. This will enable both to make efficient use of social media. The culture of using social media should spread throughout the learning community and environment. This will pave the way for learners of English as a foreign language to learn adequately and become self-learners. Curricula should be designed to cope with state of the art technology. It is necessary to apply this technology

from primary education on, so that students will be well prepared and ready to use social media wisely in learning English as a foreign language. Ongoing training is very important to cope with state of the art technology, and exchange of experience is of utmost importance in this domain.

Recommendations: In light of the results of the study the recommendations are proposed that Lecturers are encouraged to use websites as a guide to obtaining scientific material and assignments, Lecturers are encouraged to use a range of social media to develop university students' reading skills especially academic reading skills, Lecturers are invited to use social media technology to enhance other language skills inside and outside the classroom, Social media could be used as a powerful tool to meet different students' interests and learning profiles. Thus students of the same learning profiles could be engaged in similar reading activities. Along the same vein, students with the same interests could be engaged in reading similar topics and further related discussions, and Lecturers should be required to provide positive guidance for their students on how to benefit from the available social media tools to improve their reading skills.

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A Novel Efficient Deep Feature Extractor and Classifier Approach for Brain Tumor Segmentation in Magnetic Resonant Images

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ABSTRACT

The main purpose of this paper is to develop a novel deep feature retrieval approach for performing brain tumor identifying process from Magnetic Resonant images. Brain tumor is caused due to uncontrolled cell divisions. Tumor detection in the early phase is very important which is useful for diagnosis and treatment of tumor. First the input Magnetic Resonant brain image is denoised by using the Modified Decision Based Unsymmetric Trimmed Median Filter (MDBUTMF) and then the image contrast is improved with the Contrast Limited Adaptive Histogram Equalization (CLAHE). After pre-processed the input image the next step is to retrieve the features from the denoised and contrast enhanced image. To extract the features from the pre-processed image this project proposed one novel feature retrieval technique named Deep Efficient Reduced Local Derivative Pattern (DERLDP). After extracting the deep features, the next step is to partition the brain tumor based on these extracted features. To do this process the supervised segmentation approach is employed. Among several supervised segmentation approaches this work uses deep machine learning approach named Convolution Neural Network (CNN). Finally, the extracted features are given as input to these machine learning approach to partition the brain tumor regions. To find out the performance of the proposed deep feature retrieval and deep machine learning approach, four performance metrics are employed namely, Dice Similarity Coefficient (DSC), Positive Predictive Value (PPV), Jaccard Index (JI) and Sensitivity (SEN). From the experimental results, it is shown that the novel DERLDP and CNN performs better than other existing approaches.

KEY WORDS: BRAIN TUMOR, CLAHE, MDBUTMF, DERLDP AND CNN.

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INTRODUCTION

Brain tumor is a severe illness, where abnormal growths in the brain may interfere with the function of the brain. The National Fat Loss Foundation has announced that the number of persons in developing nations with brain tumors has increased by nearly 300 percent over the last three decades, (Logeswari, 2010; Lei 2016). Magnetic stereoscopic images are advanced medical image techniques that provide much data about the anatomy of soft tissue. Manual diagnosis of a brain tumor by a physician is a less-than-valid, long-term method (Menze 2014; Machhale 2015; Madabhushi 2016). The

automatic detection of brain tumors using magnetic resonant images (MRIs) aspire to categorize usual and unusual MRIs based on the nonexistence or existence of tumors correspondingly. So, diagnosing problems can be considered a challenge for image classification. The widespread brain tumor has led to large MR data creation. Thus, the evolution of a self-healing brain tumor system that receives high accuracy of tumor detection and localization is essential. Full machine learning and deep learning play an important role in brain scanning, division, registration and classification of tumor tissue, (Pan 2015; Sentas 2020).

In this section, we have propelled a few summaries of such existing studies and techniques. In Classification, the Fuzzy C-Means (FCM) segmentation is applied to separate the tumor and non-tumor region of brain. Also, wavelet feature is extracted by using multilevel Discrete Wavelet Transform (DWT). Finally, Deep Neural Network (DNN) is incorporated for brain tumor classification with high accuracy. This technique is compared with KNN, Linear Discriminant Analysis (LDA) and Sequential Minimal Optimization (SMO) classification methods. An accuracy rate of 96.97% in the analysis of DNN based brain tumor classification. But the complexity is very high and performance is very poor. New multi-fractal (MultiFD) feature retrieval and improved AdaBoost classification schemes are employed to identify and partition the brain tumor. The texture of brain tumor tissue is extracted by using MultiFD feature retrieval scheme. The improved AdaBoost classification methods are employed to find the given brain tissue is tumor or non-tumor tissue. Complexity is high. Local independent projection-based classification (LIPC) method is employed to classify the voxel of the brain (Sentas 2020).

Also, path feature is extracted in this method. Hence no need to perform explicit regularization in LIPC. The accuracy is low. In MRI Images, a seeded tumor segmentation method with new Cellular Automata (CA) technique is presented, which is compared with graph cut based segmentation method. The seed selection and Volume of Interest (VOI) is calculated for efficient brain tumor segmentation. Also, tumor cut segmentation is incorporated into this work. The complexity is low. The accuracy is also low (El-Dahshan 2014; Ahmmed 2017). Researchers have suggested a strategy that accomplishes tumor stage by utilizing ANN. The Brain Tumor arrange is ordered utilizing the ANN classifier. The precision of the proposed strategy was expected around 97.44% (Ahmmed 2017). Researchers have contrived a novel strategy for brain tumor identification that envelops Histogram Normalization and selection of K-implies/ K means Segmentation schemes (Singh et al.2016).

MRIs can be productively grouped the SVM in order to offer exact expectation and characterization. SVM classifier allegedly gave the precision of 91.49%. As obviously apparent, the SVM approach offered higher precision. Researchers have received a scholarly grouping framework to sort normal and abnormal MR brain. In the classification step, diverse machine learning strategies like SVM, KNN and SVM-KNN have been embraced and a similar report among them is encouraged (Nandpuru 2016). Researchers have come up with an algorithm that is a mix of SVM and fuzzy c-implies, a hybrid scheme for recognition of brain tumor from MR scans (Parveen 2015). Researchers uses histogram, which computes the total quantity of specified pixel values distributed in a particular image (Sarma 2012). The algorithm was tested on 48 images where the overall accuracy rates for all images were around 95%. An advanced brain tumor segmentation was introduced, which was also called multimodal brain tumor segmentation scheme (LeCun 2010).

Hybrid feature selection with ensemble classification was applied for brain tumor diagnosis process. The GANNIGMAC, decision Tree, Bagging C based wrapper approach was employed to obtain the decision rules. The DeepSeg developed by Zeineldin et al. (2020) is a modular decoupling framework. It consists of two connected core parts based on an encoding and decoding relationship. The encoder part is a convolutional neural network (CNN) responsible for spatial information extraction. The resulting semantic map is inserted into the decoder part to get the full-resolution probability map. Automated segmentation using CNN has been proposed by Bhandari et al. (2020). CNNs work by using an input, convoluting this input with a filter (also termed a kernel) and giving an output.

Wentao Wu et al. (2020) proposes a deep convolutional neural network fusion support vector machine algorithm (DCNN-F-SVM). The proposed brain tumor segmentation model is mainly divided into three stages. In the first stage, a deep convolutional neural network is trained to learn the mapping from image space to tumor marker space. In the second stage, the predicted labels obtained from the deep convolutional neural network training are input into the integrated support vector machine classifier together with the test images. The advantages of the convolutional neural network are the fact that it provides optimal accuracy of segmentation. However, this is at the cost of computational load. To overcome this drawback, this paper introduces a novel feature extracting approach (Bhandari et al. 2020).

The main contributions of this paper are summarized as follows:

- CNN classifier was applied to partition the brain tumor region from the given input MR image. CNN classifier was trained by the brain MR image dataset BRATS datasets.
- For extracting features, a novel deep feature was employed named Modified Decision Based Unsymmetric Trimmed Median Filter (MDBUTMF)
- CNN classifier was compared with PNN and ANN classifiers.
- The proposed method provided good results for BRATS data set.

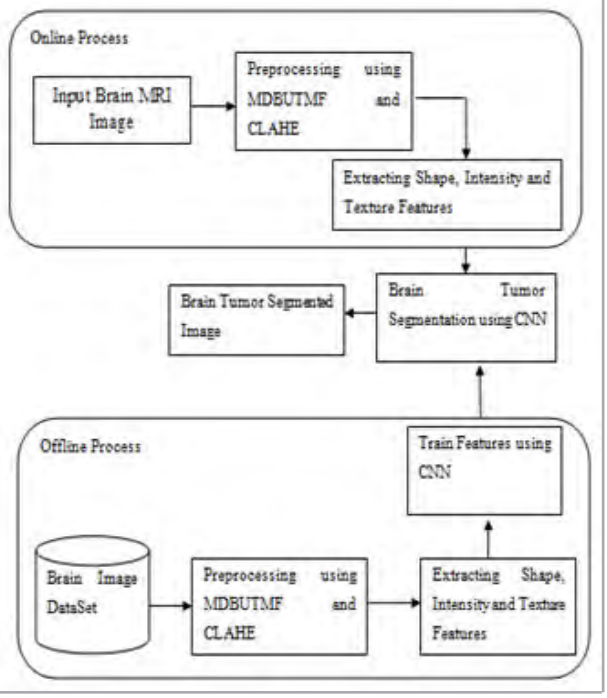
In the next section, we have proposed our methodology to identify and classify brain tumor from brain MR

images that we have deduced by overcoming the found limitations on the subject.

MATERIAL AND METHODS

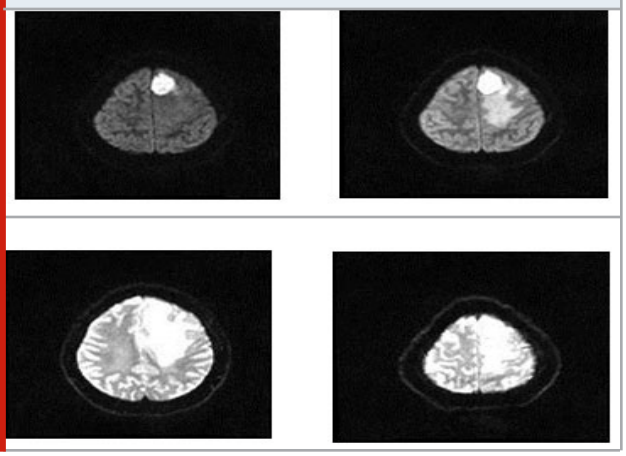
The overall architecture for the brain tumor segmentation based supervised learning has been shown in Fig. 1. First the input MR brain image is denoised by using MDBUTMF and then the image contrast is enhanced by using the CLAHE. After pre-processed the input image the next step is to extract the features from the denoised and contrast enhanced image. To extract the features from the pre-processed image this project proposed one novel feature retrieval technique named Deep Efficient Reduced Local Derivative Pattern (DERLDP). After extracting these deep features, the next step is to partition the brain tumor based on these extracted features. To do this process the supervised segmentation approach is employed. Among sever supervised segmentation approach this works uses deep machine learning approaches named CNN. Finally, the extracted features are given as input to these machine learning approaches to partition the brain tumor regions.

Figure 1: Overall Architecture of the Proposed Work



For the dataset used, the machine learning methods presented in this paper was evaluated on the BRATS 2015 databases. These datasets are downloaded from the site. A total of 4,800 images of the brain tumors were evaluated. The proposed technique is employed to the image of the tumor in the brain. During the training phase, a 4,000-millimeter brain tumor was employed, but each had 1000 pieces of brain tumors. It has five types of tumors, and each image has 200 images displayed. Experiments are carried out on a BRATS2015 FLAIR, T1, T1c and T2 training ground truth database of 4000 MR images of brain tumor and 800 testing ground truth databases. Fig. 2 depicts the sample brain tumor images from the BRATS 2015 database.

Figure 2: Experimental Images



For preprocessing, first, for the primary infrared image of the MR brain is enhanced by adaptive techniques. Analogue alignment (AHE) is a computer image processing technology employed to increase image levels. It differs from the simple histogram alignment, because many histogram manipulation methods, each corresponding to a particular part of the image, use them to distribute the light of the image. So, it is suitable for improving the local contrast and improving the edge definitions in each area of the image. However, AHE has a tendency to exaggerate noise in areas with the same image. The variant isotope variant called CLAHE, as opposed to the histogram, changes it by limiting the profit margin. Chip algorithms are shown in # 1 algorithm.

Algorithm1:

1. 1. Get al.l inputs: images, number of fields in rows and rows, histogram containers to use to create variable image function (dynamic range), limit delimation limit (normal 0 to 1)
2. 2. Prior to processing input: Set the exact limit of the clip from the normal value, if necessary, adjust the image before dividing it into the area.
3. Process each area context (tile), and be able to produce image of grayscale: the single-zone retrieval of the image, creating the graph of the area using the number of bins, the histogram snap using the set of the set Create conversion (changes function) for this area.
4. 4. Correct image of gray level, in order to collect the final image of CLAHE: extracted from clusters of four adjacent features of the image processing map area by each tile-over-part artwork, download the application Pixel of Four Map Exercises to the Pixel and Interpolate Between Results to Get This Pixel Out! Repeat the whole image.

Next, for pre-processing, Modified Decision Based Unsymmetric Trimmed Median Filter (MDBUTMF) is employed on an enhanced MR brain images for

de-noising. This filter processes corrupted images by identifying noise. The pixel process checks whether it is strong or not. That means that if the pixel runs between the maximum and minimum values of the gray level, it is considered to be in pixel, it is still unchanged. If the PPP process takes up the maximum green level, and the minimum is a puzzling pixel running by MDBUTMF.

For feature extraction, after denoising and contrast enhancement process, the next process is to retrieve the features from the preprocessed images. This work introduced one innovative approach for extracting deep features from the MR image named Deep Efficient Reduced Local Derivative Pattern (DERLDP). This approach uses the Efficient Reduced Local Derivative Pattern to extract the deep features using Convolution Neural Network (CNN). The deep features are employed to enhance the brain tumor partition accuracy. So first see the detailed explanation about Efficient Reduced Local Derivative Pattern. In the Efficient Reduced Local Derivative Pattern first take the neighbor values as mentioned in the above figure. And then apply the below formula to find the difference.

PDP= C-N (1)

In the equation 1 C is the center pixel value and N is the neighbor pixel value. And then calculate the direction by using the below formula.

If PDP1 >0 and PDP2 >0 PV = 1
PDP1<0 and PDP2 <0 PV = 2
PDP1 > 0 and PDP2 <0 PV = 3
PDP1 < 0 and PDP 2 >0 PV = 4 (2)

Then calculate the histogram of the pattern description value. Consider these histograms as the feature vector. Compare the query image with the images in the database employed. Based on the best matches retrieve the images from database. And then these features are given into the CNN. For classification, after extracting the feature, the next step is to partition the brain image based on these retrieved features using supervised segmentation approach. Among various supervised segmentation approaches this work only concentrate the machine learning approaches. From the various machine learning approaches this work takes only deep classifier named Convolution Neural Network.

Under convolution neural network, the CNN based brain tumor segmentation is divided into two phases such as training and testing. In the training phase, preprocessing, feature exaction and classification with Loss function is performed to make a prediction model. Initially, label the training image set. In the preprocessing image resizing is applied to change size of the image. Finally, the convolution neural network is employed for automatic brain tumor classification. In the proposed CNN, we will train only last layer. We don't want to train all the layers. So, computation time is low meanwhile the performance is high in the proposed automatic brain tumor classification scheme. The loss function is calculated by using gradient descent algorithm. The raw image pixel is mapping with class scores by using a score function. The quality of particular set of parameters is measured by loss function. It is based on how well the induced scores approved with the ground truth labels in the training data. The loss function calculation is very important to improve the accuracy. If the loss function is high, then the accuracy is low. Similarly, the accuracy is high, when the loss function is low. The gradient value is calculated for loss function to compute gradient descent algorithm.

Repeatedly evaluate the gradient value to compute the gradient of loss function. Algorithm for CNN based Classification is shown in Algorithm 2.

Algorithm 2
1. Apply convolution filter in first layer
2. The sensitivity of filter is reduced by smoothing the convolution filter (i.e.) subsampling
3. The signal transfers from one layer to another layer is controlled by activation layer
4. Fasten the training period by using rectified linear unit (RELU)
5. The neurons in proceeding layer is connected to every neuron in subsequent layer
6. During training Loss layer is added at the end to give a feedback to neural network.

RESULT AND DISCUSSION

The assessment of the supervised machine learning segmentation approaches, this paper employs four metrics namely Dice Similarity Coefficient (DSC), Positive Predictive Value (PPV), Jaccard Index (JI) and Sensitivity (SEN) (Bhandar et al. 2020; Wu et al. 2020). The DSC, metric calculates the overlap among the ground truth and the machine learning partition result based on Eq. (3) (Zhao 2016),

DSC = 2TP/(FP+2TP+FN) (3)

Where True Negative (TN) signifies the amount of brain tumor pixels accurately predicted. True Positive (TP) signifies the amount of non-brain tumor pixels accurately predicted. False Positive (FP) signifies to the amount of brain tumor pixels wrongly predicted as non-brain tumor pixels. False Negative (FN) corresponds to the amount of non-brain tumor wrongly predicted as brain tumor pixels. PPV is a calculated of the amount of FP and TP based on Eq. (4),

PPV = TP/(FP+FP) (4)

The Jaccard Index is calculated as the amount of the intersection of the brain tumor and non-brain tumor pixels divided by the amount of their combination based on Eq. (5),

JI = TP/(TP+FP+FN) (5)

At last, Sensitivity metric is calculated to estimate the number of TP and FN based on Eq. (6),

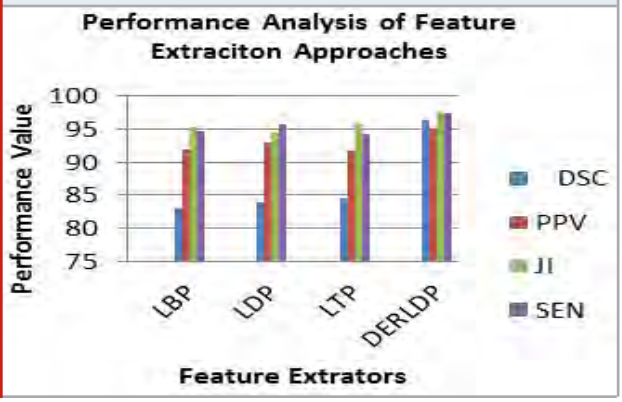
SEN=TP/(TP+FN) (6)

Table 1. Analysis of PPV, DSC, JI and SEN of BRATS 2015 Dataset for Feature Extraction Approaches

Dataset Feature Descriptor Metrics DSC	PPV	JI	SEN	
LBP	83.093	91.993	95.213	94.633
LDP	84.003	93.013	94.383	95.723
LTP	84.523	91.603	95.603	94.203
DERLDP	96.333	94.973	97.633	97.393

Experiment No 1: Analysis of Feature Extraction Approaches: In this experiment, we will evaluate the contribution of each feature retrieval techniques which are employed in the work. To assess the efficiency of this feature retrieval scheme, the PPV, DSC, JI and SEN measures are employed. It is shown in equation 3,4,5 and 6 correspondingly. Ideally, an excellent feature retrieval approach is accepted to have a high PPV, DSC, JI and SEN value. Table 1 lists the PPV, DSC, JI and SEN measures of feature retrieval approaches.

Figure 3: Analysis of PPV, DSC, JI and SEN of BRATS 2015 Dataset for Feature Extraction Approaches



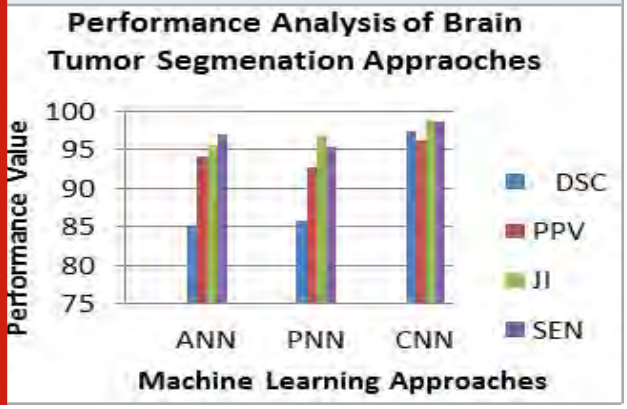
As observed from Table 1, the PPV, DSC, JI and SEN of the DERLDP in range 95-97, which is superior than that of the traditional individual feature retrieval method. So, the combined features are best for the brain tumor detection approach. Fig.3 depicted the PPV, DSC, JI and SEN measures of feature retrieval approaches (Bhandar et al. 2020; Wu et al. 2020). As observed from Fig.3, the PPV, DSC, JI and SEN of the DERLDP in range 95-96, which is superior than that of the traditional individual feature retrieval method. So, the combined features are best for the brain tumor detection approach.

Experiment No 2: Analysis of Brain Tumor Segmentation Approaches: In this experiment, this work will evaluate the contribution of each brain tumor segmentation approaches which are employed in the work. To assess the efficiency of this brain tumor partition technique, the PPV, DSC, JI and SEN measures are employed. It is shown in equation 3,4,5 and 6 correspondingly. Ideally, a good brain tumor segmentation approach is expected to have a high PPV, DSC, JI and SEN value. Table 2 lists the PPV, DSC, JI and SEN measures of brain tumor segmentation approaches (Bhandar et al. 2020; Wu et al. 2020).

Table 2. Analysis of PPV, DSC, JI and SEN of BRATS 2015 for Brain Tumor Segmentation Approaches

Dataset Machine Learning Segmentation Metrics	DSC	PPV	JI	SEN
ANN	85.212	94.222	95.592	96.932
PNN	85.732	92.812	96.812	95.412
CNN	97.542	96.182	98.842	98.602

Figure 4: Analysis of PPV, DSC, JI and SEN of BRATS 2015 for Brain Tumor Segmentation Approaches



As observed from Table 2, the PPV, DSC, JI and SEN of the CNN in range 96-97, which is superior than that of the other machine learning brain tumor segmentation approach. So, the CNN is best for the brain tumor detection approach. Fig.4 depicted the PPV, DSC, JI and SEN measures of brain tumor segmentation approaches. As observed from Fig.4, the PPV, DSC, JI and SEN of the CNN in range 96-98, which is superior than that of the other machine learning brain tumor segmentation approach. So, the CNN is best for the brain tumor detection approach. The contribution of proposed method is evaluated with the existing works. To evaluate the performance of this proposed approach, the performance metrics used are DSC, sensitivity and specificity. Ideally, a good proposed approach is expected to have a high DSC and sensitivity. The proposed method shows 96.33% DSC and 96.32% sensitivity for BRATS data set, which is superior than that of the existing works (Bhandar et al. 2020; Wu et al. 2020).

CONCLUSION

Segmenting the brain tumor is a complicated work, so the error can result in much more. This paper presented a novel deep feature retrieval approach DERLDP and deep classifier method CNN for brain tumor segmentation. The performance of this approach is estimated by PPV, DICE, Sensitivity and Jaccard. The experiments are done on BRATS 2015 dataset. This paper concluded that DERLDP with CNN method produce best result than other existing approaches. In accordance with DICE and Jaccard metrics, this paper justified that the results from DERLDP with CNN segmentation were extraordinarily like to the ground truth segmentation. With the aim of improve CNN, this paper intends to expand the no of training images as well as build up an efficient feature retrieval technique to improve the efficiency of the CNN. In this work, in certain cases, some non-tumor slices were incorrectly classified as tumor (false positive outputs). To improve the accuracy, it is possible to remove the percentage of the image (region of the skull), in the future work and thereby reducing the possibility of false positives in areas that are certainly not. The system will also be experimented in future use of 3D brain datasets.

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In vitro Anti inflammatory studies of the leaf extracts of *Litsea quinqueflora* (Dennst.) Suresh

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ABSTRACT

Drugs derived from plants have been used routinely in modern medicines, especially in the treatment of chronic disorders. Identification, isolation and characterization of active principles from plant derived drugs are of prime importance nowadays. It demands an extensive pharmaceutical study of the different extracts of medicinal plants to identify their biological properties. The present study was designed to assess the anti-inflammatory potential of *Litsea quinqueflora* (Dennst.) Suresh. Leaf paste of *L. quinqueflora* have been used by many traditional healers as a remedy for inflammatory disorders. But scientific validation of this practice still remains undone and hence an attempt has been made to evaluate the anti-inflammatory potential of different leaf extracts of *L. quinqueflora*. Dried powder of leaves was sequentially extracted with hexane, chloroform, ethyl acetate, methyl ethyl ketone, methanol and water. Preliminary phytochemical screening revealed the presence of flavonoids, steroids, terpenoids, phenols, alkaloids, resins, glycosides etc. in the various extracts. Ethyl acetate, methyl ethyl ketone and methanol extracts were selected for further studies due to the presence of most of the phytochemicals. Anti-inflammatory activity of the extracts was tested by inhibition of protein denaturation, proteinase inhibition and Human Red Blood Cell membrane stabilization assays. Percentage of inhibition and IC_{50} values were calculated. The assay concluded that ethyl acetate, methyl ethyl ketone and methanol extracts showed significant inhibition ($p \leq 0.05$) in a concentration dependent manner and there by the anti-inflammatory property. Among them methanolic extract (LM) showed highest activity. The results of this study supported the efficacy of *L. quinqueflora* as herbal anti-inflammatory agent.

KEY WORDS: LITSEA QUINQUEFLORA, PHYTOCHEMICALS, ANTI-INFLAMMATORY, PROTEIN DENATURATION, PROTEINASE INHIBITION, MEMBRANE STABILIZATION.

INTRODUCTION

The term inflammation springs out from a Latin word "inflammare" which means to burn. Inflammation is a defending process of body against noxious stimuli, infection or traumatic conditions. Inflammations can be acute or chronic with characteristic symptoms such as pain, heat, redness, swelling and loss of function (Raghavendra et al., 2015). It is an indication of injury or disease that occurs in living system and an alert to start healing process. Though it is a security measure of body, loss of proper control and its persistence in the tissues for a long time leads to various inflammatory disorders such as asthma, allergy, multiple sclerosis, systemic lupus erythematosus, arthritis, psoriasis, arthrosclerosis, diabetes, Crohn's disease, ulcerative colitis, etc.

Inflammatory responses are associated with changes in homeostatic balances of living body (Anilkumar, 2010; Raghavendra et al., 2015). Two types of anti-

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inflammatory drugs viz. steroidal and non-steroidal are available to treat inflammatory disorders. But their long term use lead to adverse side effects. Steroidal drugs such as betamethasone, prednisolone and dexamethasone cause adrenal atrophy, osteoporosis, euphoria, cataracts, glaucoma and suppression of response to infection and injury. Non-steroidal drugs inhibit both physiological and inflammatory prostaglandins and cause peptic ulcers and bronchospasm (Chaible et al., 2017). The side effects of commercial anti-inflammatory drugs prompted the need for a safer medicine with better efficacy, economic feasibility and easy availability. Thus, plants with a wide range of biologically active compounds became novel sources of crude as well as pure compounds in different disease conditions (Abdulkhaleq et al., 2018; Ogunmefun, 2018).

The investigation and identification of phytochemicals led to the development of new plant-based drugs (Abdulkhaleq et al., 2018; Ogunmefun, 2018). In the present investigation, the solvent extraction of leaves of *Litsea quinqueflora* was done successively with different solvents of non-polar to polar nature to identify the phytochemicals. Anti-inflammatory potential of leaves has been studied through different anti-inflammatory assays such as inhibition of protein denaturation, proteinase inhibition and membrane stabilization. The genus *Litsea* that belongs to the family Lauraceae is predominant in tropical and subtropical regions. Species of *Litsea* are important traditional folk medicines of those areas and sources of important secondary metabolites and essential oils. They possess various ethno pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, antitumor and cytotoxicity. There are few reports on the anti-inflammatory and antioxidant properties of *L. quinqueflora* (Anilkumar and Johny, 2015; Jose and Anilkumar, 2018; Kamle et al., 2019).

Traditional utilization of *Litsea* species in inflammatory disorders such as oedema, rheumatic arthritis and gastroenterologia have been reported (Wang et al., 2016). The recent study on antioxidant activity and DNA protective effect of fruits of *L. cubeba* is another evidence to confirm the ethnomedicinal importance of the genus *Litsea* (Seal et al., 2020). There are only very less scientific reports available on the folklore use of *L. quiqueflora* and its validation. The present study attempts to scientifically validate the anti-inflammatory properties of this plant via in vitro methods.

MATERIAL AND METHODS

Leaves of *Litsea quinqueflora* (Dennst.) Suresh were obtained from Kurianad of Kottayam district, Kerala, India. Plant specimen was identified and authenticated at Kerala Forest Research Institute, Kerala, India and voucher specimen was deposited with serial number KFRI 13057. Leaves were washed thoroughly in running water and shade dried. The dried leaves were powdered using a mechanical blender and used for extraction using different solvents. The leaf powder was extracted sequentially in a soxhlet apparatus with different solvents such as hexane, chloroform, ethyl acetate, methyl ethyl ketone, methanol and water. Each extract was collected, filtered and dried using a vacuum evaporator followed by redissolving in 5% Dimethyl sulfoxide (DMSO) and was used for further studies. Preliminary phytochemical screening to identify different classes of phytochemicals was done as per the method of Harborne (1998).

Based on the results obtained from preliminary phytochemical screening, further anti-inflammatory assays were carried out using ethyl acetate (LE), methyl ethyl ketone (LMEK) and methanol (LM) since all other extracts contained negligibly lesser number of phytochemicals. Three screening assays such as inhibition of protein denaturation, proteinase inhibition and HRBC membrane stabilization activity were done to assess the anti-inflammatory activity of different solvent extracts of *L. quinqueflora*. Inhibition of protein denaturation was done as per the method of Mizushima and Kobayashi (1968). The reaction mixture contained 0.45 ml of 1 % aqueous solution of bovine serum albumin (BSA) with 0.05 ml of different leaf extracts having concentrations (62.5, 125, 250, 500 µg/ml) and the pH was maintained at 6.3. It was incubated at 37 °C for 20 minutes and later temperature was raised up to 57 °C for 3 minutes. It was then allowed to cool at room temperature. Then added 2.5 ml of phosphate buffered saline (PBS) of pH 6.3 and measured the optical density (OD) at 660 nm using UV-Vis spectrometer (Shimadzu - UV 1800) with PBS as blank. Diclofenac sodium was used as the standard anti-inflammatory drug. Test control was mixed with distilled water instead of extract and distilled water was added instead of BSA in the product control. The percentage of inhibition of protein denaturation (Kiranmayi, 2018) was calculated by the formula,

$$\% \text{ of inhibition} = 100 - \left[\frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of test control}}\right] \times 100$$

Proteinase inhibitory activity was executed according to the method of Oyedapo et al., (1995) with slight modifications. The reaction mixture consisted of 2 ml of 20 mM Tris HCl buffer (pH 7.4), proteinase (0.06mg), and 62.5, 125, 250 or 500 µg/ml of test solution. After incubating for 5 minutes at 37°C, 1 ml of 0.8 % (w/v) casein was mixed with the reaction mixture. It was kept under incubation for 20 minutes and the reaction was terminated by the addition of 2 ml of 70 % perchloric acid. The cloudy suspension thus obtained was centrifuged at 3000 rpm for 10 minutes. The optical density of the clear supernatant was measured at 210 nm in a UV- visible spectrophotometer against Tris - HCl buffer as blank. The following formula was used to calculate the percentage of inhibition,

$$\% \text{ of inhibition} = 100 - \left[\frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of test control}}\right] \times 100$$

Where, product control was devoid of casein and the test solution and test control without the drug. Anti-inflammatory activity was also tested using human red blood cell (HRBC) membrane lysis and hypotonicity and

heat induced membrane lysis methods as per Oyedapo et al., (1995) with minor modifications. Blood sample was taken from a healthy volunteer who had not been administered any anti-inflammatory drug for the past two weeks of experiment. It was centrifuged at 3000 rpm for 10 minutes at room temperature. The red blood cells thus obtained were repeatedly washed isosaline (0.85% w/v Sodium chloride) till a colourless supernatant appeared. From the final wash, a 10% RBC suspension was prepared using isosaline.

The centrifugation and washing were continued repeatedly until the supernatant became colorless. HRBC suspension of 10% was prepared using isosaline. Hypotonicity induced hemolysis was performed by preparing a reaction mixture with 2 ml hyposaline (0.25% w/v NaCl), 1 ml 0.15M sodium phosphate buffer (pH 7.4), 0.5 ml 10 % blood cell suspension and 1 ml of extract in different concentrations (62.5,125, 250, 500 µg/ml) and the final volume was made up to 4.5 ml using isosaline. Diclofenac sodium was used as the standard drug and the reaction mixture was incubated for 45 min at 40°c. The tubes were then centrifuged at 3000 rpm for 10 min and the absorbance was measured was spetrophotometrically at 560nm. The percentage of inhibition was calculate using the formula

$$\% \text{ of inhibition} = 100 - \left[\frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of test control}}\right] \times 100$$

where, product control means the absorbance of drug without HRBC and test control contained the HRBC but not the test sample.

Heat induced hemolysis was done by preparing the reaction with 1 ml of HRBC suspension (10%) and 1 ml test samples of different concentrations (62.5, 125, 250, 500 µg/ml). Test control contained 1 ml saline instead of test sample and product control omitted HRBC. Diclofenac sodium served as standard drug. The reaction mixture was incubated at 56 ° C for 30 minutes followed by cooling under tap water. Then centrifugation was carried out at 2500 rpm for 5 min. The optical density of supernatant was measured in UV-visible spectrophotometer. Percentage of inhibition was calculated using the formula,

$$\% \text{ of inhibition} = 100 - \left[\frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of test control}}\right] \times 100$$

Statistical analysis was performed in triplicates and the results were expressed in mean with standard division. Regression analysis was done in Microsoft excel 2007 version. The inhibitory percentages obtained with each concentrations of each assays were statistically analyzed through one way analysis of variance (ANOVA) followed by Tukey's post-hoc test using IBM spss statistics 25.

RESULTS AND DISCUSSION

Results of the preliminary screening of different extracts revealed the presence or absence of different phytochemical compounds is shown in Table (1). Methanol extract contained more number of compounds such as flavonoids, coumarins, tannins, saponins, steroids, terpenoids, phenols, alkaloids, resin, carbohydrates and reducing sugar or glycosides when compared to others. This can be correlated with the earlier study of Anilkumar and Johny in *L. quinqueflora* (Anilkumar and Johny, 2015). LM, LMEK and LE contained most of the phytochemicals tested when compared with hexane, chloroform and water.

Table 1. Preliminary Phytochemical Screening

Components	Hexane	Chloroform	Ethyl acetate	Ethyl methyl ketone	Methanol	Water
Flavonoids	-	-	+	+	+	-
Coumarins	-	-	+	-	+	-
Tannins	-	-	+	+	+	+
Saponins	-	+	+	-	+	-
Steroids/terpenoids	-	-	-	+	+	+
Phenols	-	-	+	+	+	-
Alkaloids	-	-	+	+	+	-
Quinines	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-
Protein	-	-	-	-	-	-
Carbohydrates	+	+	+	+	+	+
Resin	-	-	-	+	+	-
Reducing sugar/ Glycosides	-	-	+	+	+	-

Preliminary phytochemical screening of leaves of *L. monopetala* showed the presence of same compounds that exhibited pharmacological properties such as anti-microbial and anti-inflammatory (Ali-Ahmmad et al., 2012). LM, LMEK and LE are selected for the further anti-inflammatory studies because of the presence of flavonoids, phenols and alkaloids than other extracts. The medicinal properties of *Litsea* species can be attributed to the secondary metabolites present in it (Kamle et al., 2019).

Inflammation can be accompanied with gradual denaturation of protein. Here, a protein candidate has been subjected to heat induced denaturation by applying 57° C. The capacity of samples to inhibit protein denaturation was evaluated and shown in figure (1). LE showed inhibition against protein denaturation at an IC 50 value of 390.73 µg/ml as obtained from the linear curve with regression equation y = 0.082x + 17.96; R2 = 0.637. In the case of LMEK the IC 50 value was observed at 248.6µg/ml with regression equation y = 0.114x + 21.66; R2 = 0.783. LM exhibited an IC 50 value of 114.8 µg/ml from the linear curve with regression equation y = 0.075x + 41.39; R2 = 0.919. From this it was clear that LM has more inhibitory activity than that of LE and LMEK. Percentage of inhibition of all the three extracts was significant ($p \leq 0.05$) on a dose dependent manner.

Highest inhibitory percentage of LM is 76.7 % at 500µg/ml, LMEK at 500µg/ml is 72.51% and that of LE is 53.55%. The IC50 values of leaf extracts of *Aegle marmelos* and *Ocimum sanctum* in protein denaturation assay were 95.64µg/ml and 42.17µg/ml respectively (Reshma and Brindha, 2014) and is an indication of its higher activity than that of LM, LMEK and LE. The results obtained in *Polyalthia longifolia* and *Pergularia daemia* (Iffath Hina and Rose, 2018; Ogbomade et al., 2019) can be compared with that of *L. quinqueflora*. In a study using ethanolic leaf extract of *Nauclea latifolia* Sm (Iheagwam et al., 2020), the highest activity of 70.54 % was observed at 5 mg/ml.

From this it is clear that *L. quinqueflora* has greater inhibition of protein denaturation than most of the previously reported similar cases. All the three extracts used in the present study showed significant inhibitory activity against proteinase action when compared with standard diclofenac sodium as shown in figure (2). The IC 50 value of LE was 324.18 µg/ml (y = 0.0914x + 20.50; R2 = 0.87), LMEK was 215.39 µg/ml (y = 0.089x + 30.83; R2 = 0.762) and that of LM was 122.21µg/ml (y = 0.086x + 39.49; R2 = 0.868). Statistically, all the three extracts showed increase in percentage of inhibition with increase in concentration and each concentration levels were significantly different ($P \leq 0.05$). The proteinase inhibitors have significant role in inflammation. Inflammation caused the infiltration of neutrophils which can be a rich source of serine proteases. Intracellular release of these proteases causes damage of microbes. But extracellular release leads to tissue damage during inflammatory conditions (Pham, 2008). The alcoholic leaf extract of *Justicia gendarussa* inhibit proteinase activity at 209µg/ml which was less than that of LM and thus noticed higher activity of LM. But the IC50 value of root extract of *J. gendarussa* showed inhibition at 54µg/ml and higher activity than that of LM, LMEK and LE (Patel and Zaveri 2014; Naz et al., 2017).

The IC50 value in proteinase inhibition of methanolic extract of *Aristolochia indica, Cuscuta pedicellata, Melilotus indicus* and *Tribulus terrestris* were higher than that of present extracts of *L. quinqueflora* (Naz et al., 2017). The IC50 value of leaf extracts of *Moringa oleifera* (Saleem et al., 2020) was higher than that of LMEK and LM. Hence the study revealed that all the three extracts were able to inhibit trypsin or achieve 50% of inhibition with less amount of extract when compared with lesser concentration of already mentioned plants. In membrane stabilization assay, HRBC membrane acts as an imitation of lysosomal membrane. Lysosomal membrane is significant in inflammation as its breakage causes infiltration of various inflammatory mediators into the inflammatory site. Accumulation and persistence of these intracellular lysosomal constituents gradually lead to chronic inflammation. A drug which is able to prevent breakage of HRBC membrane can contribute to the stability of lysosmal membrane and thus can be considered as an anti-inflammatory agent. Here, HRBC was exposed to heat and hypotonicity as two stimulants in order to create an inflammatory condition (Amrutkar et al., 2017).

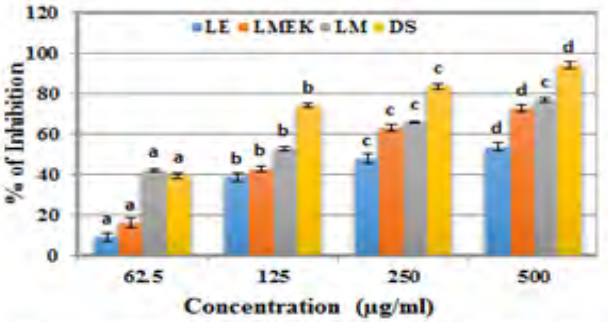
In both conditions, membrane started to lyse and as a result of lysis, haemoglobin leaks out. The amount of haemoglobin came out the cell due to cell lysis is proportionate to the grade of lysis. The samples exhibited effective inhibition against hypotonicity induced hemolysis as showed in figure (3) and was significant with an IC50 value of 340.28 µg/ml (y = 0.124x + 7; R^2 = 0.926) for LE. LMEK showed 276.1 µg/ml as IC 50 value (y = 0.131x + 13.83; R^2 = 0.935) and LM as 137.4 µg/ml (y = 0.073x + 39.97; R^2 = 0.762). Membrane stabilization property of direct methanolic extract of leaves of *L. quinqueflora* has been studied and reported where 400µg/ml exhibited a 60% inhibition (Anilkumar and Johny, 2015). Here, the sequentially extracted LM shows 61.89% at 250µg/ml. This results point to the fact that LM obtained via sequential extraction is more purified and potentially active than that of the crude one.

Blood samples or HRBC membrane is intact in isosaline conditions. When placed in hypotonic solution, osmotic stress results thus causing gradual lysis of the outer membrane to ooze out haemoglobin. The inhibitory potential of sample can be compared with that of ethanolic leaf extract of *Dalbergia sissoo* which exhibited a similar level of inhibition (Amrutkar et al., 2017). The study of methanolic leaf extract of *Mucuna pruriens* expressed less inhibition (Anosike et al., 2019) when compared with that of LM.

LE, LMEK and LM could effectively inhibit hemolysis at each concentration level. The results obtained after heat induced HRBC lysis is shown in figure (4). LE showed

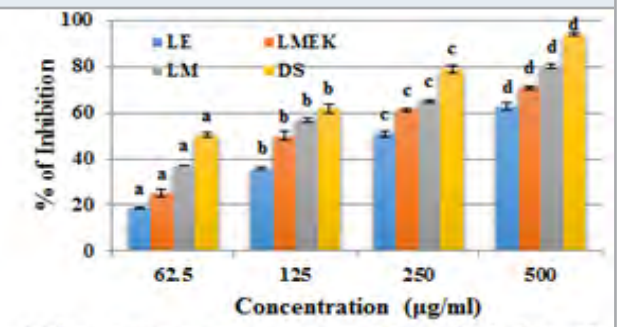
an IC 50 value of 389.15 µg/ml (y = 0.094x + 13.42; R^2 = 0.875), LMEK at 282.73 µg/ml (y = 0.099x + 22.01; R^2 = 0.792) and that of LM at 127.93 µg/ml (y = 0.087x + 38.87; R^2 = 0.792).When HRBC membrane is exposed to an elevated temperature, thermal osmolysis can occur. It leads to swelling of cells due to the increase in turbidity of suspending solution. The rapid increase in temperature and swelling causes slow rupture of cell membrane and driving out of cell constituents. This prolonged hemolysis diverts the normal effect of inflammation into inflammatory disorders. This assay enables the measurement of inhibition of hemolysis by the leaf extract of *L. quinqueflora* and thus inflammation (Tsong and Kingsley, 1975). Methanolic extract of whole plant of *Enicostemma axillare* (Leelaprakash and Dass, 2011) also effectively inhibit heat induced hemolysis and less potent than LM (Kumar et al., 2011).

Figure 1: Inhibition of protien denaturation at different concentration of extract.



Values represent mean ± standard deviation of triplicate determination. Column with different letter showed significant difference (P≤0.05).

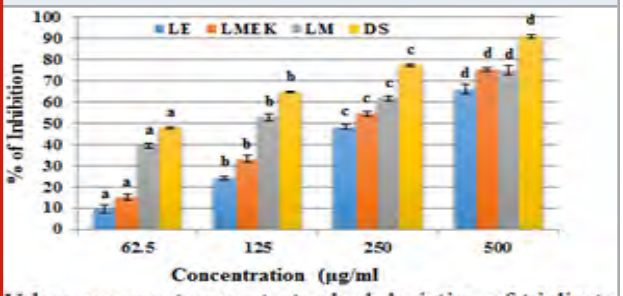
Figure 2: Proteinase Inhibition at different of extract.



Values represent mean ± standard deviation of triplicate determination. Column with different letter showed significant difference (P≤0.05).

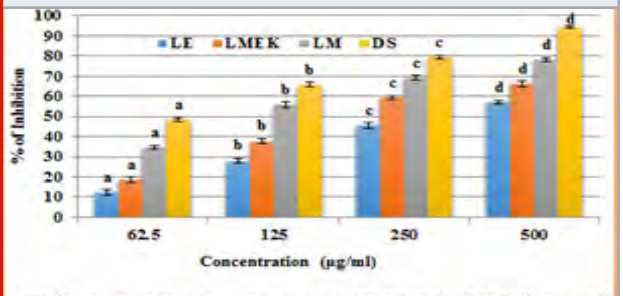
The inhibitory percentage of leaf extracts of *Baselia alba* against membrane lysis is almost similar to that of LE but less than that of LMEK and LM (Kumar et al., 2011). The membrane stabilization activity of *Vitex leucoxylon* showed highest percentage of inhibition with lowest concentration and the inhibitory activity of its alcoholic extracts are higher than that of *L. quinqueflora* extracts (Faimum et al., 2013). Gunathilake et al reported heat induced membrane stabilization assay in methanolic extracts of *Cassia auriculata, Passiflora edulis, Sesbania grandiflora, Olax zeylanica, Gymnema lactiferum* and *Centella asiatica* (Gunathilake et al., 2018). Though they inhibit hemolysis, its percentage activity is less when compared to LM, LMEK and LE. In most of the earlier studies, anti-inflammatory screening assays were reported using methanol extract of plant parts. Here, ethyl acetate, methyl ethyl ketone and methanolic extracts were used for each assay. Methyl ethyl ketone was preferred to avoid hindrance due to mucilage. All of them showed significant inhibition (p≤0.05) on a dose dependent manner. Their inhibitory percentage is directly proportional to increase in concentration. Methanolic extract showed highest activity when compared with LMEK and LE (Faimum et al., 2013).

Figuire 3: Inhibition of hypotonicity induced hemolysis at different concentration of extract.



Values represent mean ± standard deviation of triplicate determination. Column with different letter showed significant difference (P≤0.05).

Figure 4: Inhibition of heat induced hemolysis at different concentrations of extract.



Values represent mean ± standard deviation of triplicate determination. Column with different letter showed significant difference (P≤0.05).

CONCLUSION

This present study supported the use of *L. quinqueflora* as a traditionally used medicinal plant against inflammatory disorders. Though there are scanty reports available on the bioactivity of this plant, it has gained importance as a local remedy against inflammation. This study thus revealed its potency as an anti-inflammatory agent

by inhibiting protein denaturation, proteinase activity and membrane lysis. The presence of various classes of phytochemicals contributes to its pharmacological properties. Further studies to evaluate the pharmacological activities of the isolated components of *L. quinqueflora* are under progress in our lab.

Conflict of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Physico-Chemical and Microbial Characterization of Soil Collected from Pesticides Infused Industrial Area, Gujarat Industrial Development Corporation (GIDC) Naroda, Ahmedabad, Gujarat

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ABSTRACT

Pesticides are chemical substances applied to mitigate the agricultural and domestic pests, but indiscriminate and unsafe use leads to their accumulation in the environment. Bioaccumulation of pesticides is of great public health concern due to their toxicity. The enhanced production and formulation of pesticides has posed serious problem through contaminating the nearby surroundings, which ultimately affect the biological diversity. Therefore, the present study is focussed on physico-chemical and microbial characterization of pesticides contaminated industrial soil nearby pesticides industry. For this, the soil sample was collected from nearby pesticides industries from GIDC Naroda, Ahmedabad district of Gujarat state. The soil sample was analysed for various physico-chemical characteristics such as temperature, pH, electrical conductivity, moisture contents, water holding capacity, bulk density, hardness, chloride, alkalinity, sulphate, available phosphorus, total phosphorus, nitrate, nitrite, ammonium, total organic carbon and total organic matter. For microbial characterization, isolation of bacteria was carried out using serial dilution pour plate technique and characterized morphologically, biochemically. The molecular characterization of the bacteria isolates was done by 16S rRNA sequencing. For this genomic DNA was extracted using CTAB method and amplified. The amplified DNA was sequenced for identification of bacteria. The isolated bacteria were identified as *Bacillus licheniformis, Bacillus subtilis, Bacillus* sp., *Bacillus thuringiensis*, *Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus velezensis, Kocuria flava, Pseudomonas* sp. and *Bacillus cereus*. The obtain results infers that pesticides contaminated soil contains diversified bacterial species. As these bacterial species are growing in the pesticides contaminated soil and can be resistant to the toxicity of the pesticides, therefore, they may be the potential candidate for the removal of that compounds for environmental clean-up.

KEY WORDS: PESTICIDES, INDUSTRIAL SOIL, MICROBIAL CHARACTERISATION, 16S rRNA SEQUENCING.

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INTRODUCTION

Pesticides are natural or synthetic compounds that are poisonous and can kill pests including insects, nematodes and rodents etc. Over the past few decades, pesticides and other agrochemicals have become a vital component of modern agricultural system, leading to a substantial improvement in crop productivity by controlling insects and other diseases (Carvalho 2017; Ali et al. 2019). The

state like Gujarat, agriculture is one of the most important sector as it is the primary sources of livelihood for more than half (~ 60%) of its workforce (Planning Commission 2004; UNDP 2004). Currently, huge amount of pesticides are being used in agricultural field for protection of crops from the pests. Due to increasing demand of pesticides, a lots of manufacturing and formulation industries has been established in Gujarat to fulfil the requirements (Morillo and Villaverde 2017; Varjani et al. 2018).

On the other hand, enhanced Industrial and agricultural activities in recent years, in India especially in Gujarat has led to considerable contamination of air, soil and groundwater resources due to release of large amounts of pesticides and other chemicals in the environment (Morillo and Villaverde 2017; Varjani et al. 2018). Indiscriminate use of pesticides for pest infestation and vector control has drawn special attention of scientific community globally due to the multifaceted toxicity, mobility, persistence and recalcitrant properties (Fantke and Jolliet 2016; Liu et al. 2016; Varjani et al. 2018).

The rapid increase in pesticides industries also has polluted the nearby environment severely. Nevertheless, these pesticides possesses several negative impacts ranging from ecological pollution to damage of biodiversity (Pico et al. 2018; Barbieri et al. 2019; Köck-Schulmeyer et al. 2019). This can influences the physico-chemical properties and microbial diversity of the soil and possibly can cause a threat to both the environment and human health (Samant et al. 2018). Although, some studies show physico-chemical and microbial characterization of pesticides contaminated industrial soil from different industrial area of Gujarat (Doolotkeldieva et al. 2018; Ravi et al. 2019), but pesticides infused industrial soil of GIDC Naroda, Ahmedabad, Gujarat is yet to be studied. Therefore, the present study was carried out to characterize the pesticide infused soil from Gujarat for their physico-chemical analysis and microbial diversity.

MATERIAL AND METHODS

The soil sample was collected randomly from five sites from nearby pesticides industries from Gujarat Industrial Development Corporation (GIDC), Naroda, Ahmedabad district (23°06'02"N - 72°42'14"E) of Central Gujarat, in sterilized polyethene zipper bag using auger up to a depth of 1- 15 cm and stored at 4 °C for further analysis (Jayashree and Vasudevan 2006). The important physico-chemical properties of the soil, viz. temperature, pH, electrical conductivity, moisture contents, water holding capacity, bulk density, hardness, chloride, alkalinity, total organic carbon, total organic matter, sulphate, nitrate, nitrite, ammonium, available phosphorus and total phosphorus were analysed using standard methods (APHA1998). All the physico-chemical properties of the soil were analyzed in triplicates and their mean and standard deviation (SD) was calculated. The obtained values were described as Average ± SD. The Isolation of indigenous bacteria from pesticide contaminated soil was performed by serial dilution pour plate technique using nutrient agar medium. Well grown bacterial colonies were picked and further purified by streaking. The colonies were characterized morphologically, biochemically and identified by 16S rRNA techniques. The colonies were counted and the average number of colonies per three plates was determined for CFU (colony forming unit) count.

Morphological Characterization of isolated bacterial species: The isolated pure bacterial species were grown on nutrient agar medium and examined morphologically for their shape, size, margin, constancy, elevation, opacity, and pigmentation. The gram staining test was carried out by Gram's Method using a Gram staining kit and observed under a microscope for colour and shape.

Biochemical Characterization of isolated bacterial species: The isolated bacterial species were analyzed for various biochemical properties such as motility, starch hydrolysis, catalase, oxidase, urease, indole production test, nitrate reduction test, citrate utilization test, xylose, maltose, fructose, dextrose, trehalose, inositol, sucrose, L-arabinose, glycerol, melezitose, lactose, esculin hydrolysis, amylase, gelatinase, methyl red (MR) test, vogus-proskauer (VP) test, etc. using KB001 Biochemical Test Kit (HI Media, India).

Identification of bacterial species using 16S rRNA sequencing techniques: The identification of bacterial species was carried out by 16S rRNA gene sequencing. For this genomic DNA was extracted using the CTAB method. 1 ml of well grown broth culture was centrifuged at 8000 rpm for 10 min. The pellet was resuspended in 200 µl of DW and 200 µl of buffer saturated phenol was added and incubated at 60°C for 1 hour and centrifuged at 8000 rpm for 5 min. Again 400 µl of chilled ethanol was added to the aqueous stage of DNA precipitation. The precipitated DNA pellet was washed with 70% ethanol and resuspended in nuclease-free water (Das et al. 2019). The extracted DNA was used for amplification of the 16S rRNA gene sequence with primer A109 (F) AC (G/T) GCTCAGTAACACGT and 1510 (R) GGTTACCTTGTTACGACTT (Birbir et al. 2007, Mani et al. 2012). The PCR reaction combination contained 10X Taq buffer, 2 mM MgCl2, 10 mM of dNTPs, 10 µM of each primer, 1 µl of DNA, 2U Taq Polymerase. The reaction was initiated by denaturation at 94°C for 5 min followed by 35 cycles of denaturation, annealing, and elongation. The reaction was terminated after a final elongation. The amplified product was exposed to electrophoresis on a 1.5 % agarose gel. The refined product was sequenced bidirectionally using an automated DNA sequences.

Construction of the molecular phylogenetic tree: The phylogenetic tree was constructed using 16S rRNA sequences of isolated bacteria in FASTA format. The sequences closely related to the bacteria of the present study were recovered from the NCBI and aligned using ClustalW. The phylogenetic tree was prepared using the MEGA software version 7.0 and the Maximum Likelihood

method (Tamura and Nei 1993, Kumar et al. 2016). All the sequences were deposited to the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Physico-chemical characteristics of soil collected from GIDC Naroda, Ahmedabad, Gujarat: The physico - chemical properties such as temperature, pH, electrical conductivity, moisture contents, water holding capacity, bulk density, chloride, alkalinity, hardness, sulphate, available phosphorus, total phosphorus, all types of nitrogen (nitrate, nitrite, ammonium), total organic carbon and total organic matter of the soil samples collected from GIDC Naroda of Ahmedabad district, Gujarat was carried out as shown in Table 1, which infers temperature (°C), pH, electrical conductivity (µs cm -1) value of 26.12 ± 0.13, 8.24 ± 0.13, 121 ± 27.65 respectively. The moisture content (%) and water holding capacity (%) was observed 15.85 ± 1.13 and 37.45 ± 0.82 respectively. The present results are supported by earlier studies in the case of rice field contaminated with pesticides (Raman Kumar Ravi et al. 2015) and pesticides contaminated industrial soil (Ravi et al. 2019).

Table 1. Physico-chemical Analysis of soil samples collected from GIDC Naroda, Ahmedabad

Parameters	Site 1	Site 2	Site 3	Site 4	Site 5	Average ± SD
Temperature (°C)	26.1	26	26.5	26.2	26.3	26.12 ± 0.13
pH	8.28	8	8.31	8.3	8.3	8.24 ± 0.13
Electrical Conductivity (µs cm $^{-1}$)	99	104	101	159	142	121 ± 27.65
Moisture contents (%)	14.75	14.76	16.15	17.44	16.13	15.85 ± 1.13
Water Holding Capacity (%)	37.07	37.46	36.86	36.86	37.02	37.45 ± 0.82
Bulk Density (g/ml)	1.75	1.75	1.74	1.71	1.70	1.73 ± 0.02
Chloride (mg/kg)	283	216.5	200	266.5	216.5	236.5 ± 36.04
Alkalinity (mg/kg)	200	190	230	195	200	203 ± 15.65
Hardness(mg CaCO3/kg)	64	60	66	56	54	60 ± 5.10
Sulphate (mg/kg)	4.43	5.39	4.21	2.15	3.38	3.91 ± 1.22
Available Phosphate (mg/kg)	0.84	0.58	0.92	1.06	0.93	0.87 ± 0.18
Total Phosphorus (mg/kg)	9.08	8.61	7.73	7.66	8.40	8.30 ± 0.60
Nitrate (mg/kg)	11.21	9.55	10.05	11.11	10.73	10.53 ± 0.71
Nitrite (mg/kg)	0.11	0.09	0.08	0.09	0.10	0.09 ± 0.01
Ammonium (mg/kg)	2.09	1.30	2.23	2.17	1.91	1.94 ± 0.38
Total Organic Carbon (%)	1.2	1.05	1.2	1.35	0.9	0.9 ± 0.17
Total Organic Matter (%)	1.64	1.70	1.65	1.69	1.68	1.67 ± 0.03

Note: SD – Standard Deviation

The total organic carbon and total organic matter are the significant property of soil and play important role in its fertility (Yennawar et al. 2013; King et al. 2020). The result obtained of total organic carbon (%) is 0.9 ± 0.17, whereas total organic matter (%) is 1.67 ± 0.03. The recorded results for bulk density (g/ml), chloride (mg/kg), alkalinity (mg/kg), hardness (mg CaCO3/kg), sulphate (mg/kg), available phosphorus (mg/kg) and total phosphorus (mg/kg) was 1.73 ± 0.02, 236.5 ± 36.04, 203 ± 15.65, 60 ± 5.10, 3.91 ± 1.22, 0.87 ± 0.18 and 8.30 ± 0.60 respectively. The different nitrogen contents are 10.53 ± 0.71 for nitrate, 0.09 ± 0.01 for nitrite and 1.94 ± 0.38 for ammonium nitrogen. The result obtained is supported by earlier research performed by Baishya and Sarma 2014, where slight greater value of nitrate was found, and slight difference in the nitrite and ammonical nitrogen value as reported earlier by Jia and Conrad 2009. These findings are also supported by previous studies for pesticides contaminated industrial soil (Ravi et al. 2019; Meena et al. 2020).

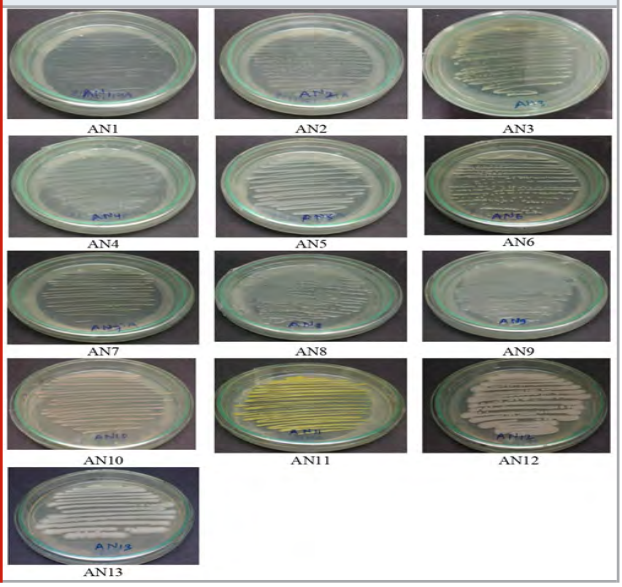
Microbiological Characterization of soil collected from GIDC Naroda, Ahmedabad (Central Gujarat): The bacterial species were isolated from contaminated soil using serial dilution pour plate technique on nutrient agar medium. A total of 13 bacterial species were isolated and pure cultured from collected soil sample of GIDC Naroda, Ahmedabad, Central Gujarat (Fig. 1).

Morphological characterization of isolated bacterial species: The isolated pure culture of bacteria was characterized by several morphological properties like shape, size, margin, elevation, pigmentation, optical character, surface, and consistency by growing them on nutrient agar plates (Table 2).

Biochemical characterization of bacterial species: Several biochemical analyses were carried out to describe the isolated bacterial species. All the bacterial species were found gram-positive except isolate AN12 (*Pseudomonas* sp. strain RKRAN12) (Table 3). All the

bacterial species were rod-shaped except isolate AN11 (*Kocuria flava* strain RKRAN11). All the bacterial species were negative for urease, starch hydrolysis, lactose, xylose, galactose, raffinose, sodium gluconate, inositol, dulcitol, arabitol, erythritol, α-methyl -D glucoside, rhamnose, α-methyl- D mannoside, xylitol, esculin hydrolysis, malonate utilization and sorbose test, while positive for catalase and dextrose test. The isolate AN2 (*Bacillus subtilis* strain CAKDS3), isolate AN4 (*Bacillus* sp. strain AN4), isolate AN5 (*Bacillus thuringiensis* strain RKRAN5), isolates AN9 (*Bacillus velezensis* strain RKRAN9), isolate AN11 (*Kocuria flava* strain RKRAN11) and isolate AN12 (*Pseudomonas* sp. strain RKRAN12) were able to reduce nitrates.

Figure 1: Bacteria isolated from GIDC Naroda soil, Ahmedabad



The isolate AN2 (*Bacillus subtilis* strain CAKDS3), isolate AN3 (*Bacillus* sp strain Y174007), isolate AN4 (*Bacillus* sp. strain AN4), isolate AN7 (*Bacillus pumilus* strain RKRAN7), isolate AN9 (*Bacillus velezensis* strain RKRAN9), isolate AN10 (*Bacillus* sp. strain RKRAN10) and isolate AN11 (*Kocuria flava* strain RKRAN11) were found positive for oxidase test. The isolate AN3 (*Bacillus* sp strain Y174007) and isolate AN10 (*Bacillus* sp. strain RKRAN10) were able to utilize citrate and adinitol, therefore positive for that test. The isolate AN1 (*Bacillus licheniformis* strain A28), isolate AN6 (*Bacillus subtilis* strain RKRAN6), isolate AN8 (*Bacillus amyloliquefaciens* strain RKRAN8) and isolate AN13 (*Bacillus cereus* strain RKRAN13) were positive for maltose, methyl red, sucrose, trehalose and melibiose test.

Identification of bacterial species by 16S rRNA gene sequencing technique: Identification of bacterial species was done by 16S rRNA gene sequencing technique. The pesticides contaminated soil show diversity of bacterial species such as *Bacillus licheniformis* strain A28, *Bacillus subtilis* strain CAKDS3, *Bacillus* sp strain Y174007, *Bacillus* sp. strain AN4, *Bacillus thuringiensis* strain RKRAN5, *Bacillus subtilis* strain RKRAN6, *Bacillus pumilus* strain RKRAN7, *Bacillus amyloliquefaciens* strain RKRAN8, *Bacillus velezensis* strain RKRAN9, *Bacillus* sp. strain RKRAN10, *Kocuria flava* strain RKRAN11, *Pseudomonas* sp. strain RKRAN12 and *Bacillus cereus* strain RKRAN13 (Fig.1). All the sequences of bacterial species (AN1 to AN13) were submitted in NCBI under the accession number MH806397, MH806398, MH806399, MK182800, MK215844, MK215849, MK215850, MK215851, MK229344, MK229345, MK229346, MK229347 and MK229348 respectively (Table 4).

Table 2. Morphological characterization of bacteria isolated from GIDC Naroda soil, Ahmedabad

Isolates	Shape	Size	Margin	Elevation	Pigment	Optical character	Surface	Consistency
AN1	Irregular	Large	Undulate	Flat	Whitish	Opaque	Smooth	Moist
AN2	Elliptical	Intermediate	Undulate	Flat	Whitish	Opaque	Smooth	Moist
AN3	Round	Large	Undulate	Convex	Whitish	Opaque	Smooth	Moist
AN4	Round	Intermediate	Entire	Convex	Creamy	Sebaceous	Smooth	moist
AN5	Elliptical	Intermediate	Curled	Unbonate	Creamy	opaque	Smooth	moist
AN6	Round	Small	Entire	Convex	White	sebaceous	Smooth	moist
AN7	Round	Small	Entire	Convex	Yellowish	sebaceous	Smooth	moist
AN8	Round	Intermediate	Entire	Convex	Yellow	opaque	Smooth	moist
AN9	Round	Small	Entire	Convex	Orange	opaque	Smooth	moist
AN10	Round	Intermediate	Entire	Convex	Creamy	Sebaceous	Smooth	moist
AN11	Round	Small	Entire	Convex	Yellowish	sebaceous	Smooth	moist
AN12	Round	Intermediate	Entire	Convex	Pinkish	opaque	Smooth	moist
AN13	Round	Small	Entire	Convex	Orange	opaque	Smooth	moist

Molecular Phylogenetic tree: The 16S rRNA sequences of bacterial species were studied to define the relationship between development and nomenclature using a phylogenetic tree. The partial sequences of the 16S rRNA gene from the isolated bacterial species (AN1 to AN13) were associated to find the closest match

using the Basic Alignment Search Tool (BLAST). The BLAST analysis revealed that the genes showed 100% similarity to *Bacillus licheniformis* (MH806397:AN1), 100 % homology to *Bacillus subtilis* (MH806398:AN2), 100 % similarity to *Bacillus* sp (MH806399:AN3), 100 % homology to *Bacillus* sp. (MK182800:AN4), 100 % similarity to *Bacillus thuringiensis* (MK215844:AN5), 100 % homology to *Bacillus subtilis* (MK215849: AN6), 100 % similarity to *Bacillus pumilus* (MK215850:AN7), 100 % homology to *Bacillus amyloliquefaciens* (MK215851:AN8), 100 % similarity to *Bacillus velezensis* (MK229344:AN9), 100 % homology to *Bacillus* sp. (MK229345:AN10), 100 % similarity to *Kocuria flava* (MK229346:AN11), 100 % homology to *Pseudomonas* sp. (MK229347:AN12) and 99.88 % similarity to *Bacillus cereus* (MK229348: AN13). The construction of a molecular phylogenetic tree was done by aligning the sequences of bacterial species.

Table 3. Biochemical characterization of isolated bacteria from GIDC Naroda soil, Ahmedabad

Tests	AN1	AN2	AN3	AN4	AN5	AN6	AN7	AN8	AN9	AN10	AN11	AN12	AN13
Gram stain	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Cocci	Rods	Rods
CFU counts													
(N x 105 cfu/g)	1.90	1.41	2.15	1.65	2.42	1.12	1.12	2.01	1.48	2.30	1.69	2.72	1.56
Motility test	-	-	+	+	-	+	-	-	-	+	+	-	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	-	+	+	+	-	-	+	-	+	+	+	-	-
Urease test	-	-	-	-	-	-	-	-	-	-	-	-	-
Indoletest	-	-	+	-	-	+	-	-	-	+	-	-	+
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	-	+	+	-	-	-	+	-	+	+	-
Methyl Red test	+	+	+	+	+	+	-	+	+	+	+	+	+
V-P test	-	+	+	+	-	+	+	-	+	+	+	-	+
O-F test	O-/F-	O+/F-	O-/F-	O-/F-	O+/F+	O-/F-	O-/F-	O-/F-	O+/F-	O-/F-	O-/F-	O+/F+	O-/F-
Triple Sugar Iron	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Glucose	+	+	+	+	+	+	V	+	+	+	+	+	+
Sorbinol	V	-	-	-	-	-	-	V	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	-	+	+	+	+	+	+	-	+	+	+	+
Fructose	+	+	-	+	+	-	-	+	+	-	+	+	-
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	+	-	+	+	+	+	+	+	-	+	+	+	+
Melibiose	+	-	-	-	-	+	-	+	-	-	-	-	+
Sucrose	+	+	-	+	+	+	-	+	+	-	+	+	+
L-Arabinose	-	-	-	-	+	-	-	-	-	-	-	+	-
Mannose	-	+	-	+	+	+	-	-	+	-	+	+	+
Inulin	-	-	-	-	+	-	-	-	-	-	-	+	-
Sodium gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	+	+	-	-	+	-	-	+	+	-	-	+
Salicin	-	-	+	-	+	+	-	-	-	+	-	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	+	-	-	+	-	+	-	+	-	-	+	-
Adonitol	-	-	+	-	-	-	-	-	-	+	-	-	-

Continue Table 3

Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-
α-methyl D	-	-	-	-	-	-	-	-	-	-	-	-	-
glucoside													
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	+	-	+	+	-	-	-	+	-	+	+
Melezitose	+	-	-	-	+	-	-	+	-	-	-	+	-
α-methyl- D-	-	-	-	-	-	-	-	-	-	-	-	-	-
mannoside													
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-
ONPG	+	-	-	-	-	-	-	+	-	-	-	-	-
Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Arabinose	-	-	-	-	+	-	-	-	-	-	-	+	-
Citrate utilization	-	-	+	-	-	-	-	-	-	+	-	-	-
Malonate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-

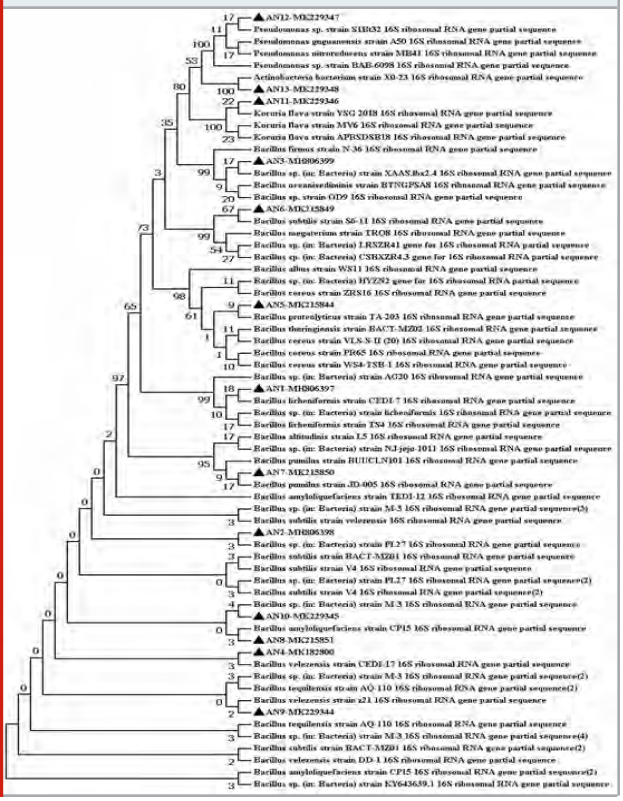
Note: (+ = Positive, - = Negative, V= 11-89% positive, O+/F-= only oxidative; O+/F+ = Oxidative and fermentative; O-/F- = glucose not metabolised; A/A= Glucose, lactose & sucrose fermentation; K/A = Glucose fermentation; K/ K = Non fermentative)

Table 4. Identification of isolated bacteria from soil

Isolates	Identification	Accession Number
AN1	*Bacillus licheniformis* strain A28	MH806397
AN2	*Bacillus subtilis* strain CAKDS3	MH806398
AN3	*Bacillus* sp strain Y174007	MH806399
AN4	*Bacillus* sp. strain AN4	MK182800
AN5	*Bacillus thuringiensis* strain RKRAN5	MK215844
AN6	*Bacillus subtilis* strain RKRAN6	MK215849
AN7	*Bacillus pumilus* strain RKRAN7	MK215850
AN8	*Bacillus amyloliquefaciens* strain RKRAN8	MK215851
AN9	*Bacillus velezensis* strain RKRAN9	MK229344
AN10	*Bacillus* sp. strain RKRAN10	MK229345
AN11	*Kocuria flava* strain RKRAN11	MK229346
AN12	*Pseudomonas* sp. strain RKRAN12	MK229347
AN13	*Bacillus cereus* strain RKRAN13	MK229348

The present study show that slight variation in the physico-chemical properties of soil around the pesticides industry are occurred, which provide better environmental condition for the bacterial diversity to grow. The study show that the soil around the pesticides industry of GIDC, Naroda, Ahmedabad district contains diversity of bacterial species. The soil is mainly dominated by different species of genus *Bacillus*. This finding was also supported by earlier studies for the soil contaminated with pesticides (Doolotkeldieva et al. 2018; Ravi et al. 2019).

Figure 2: Phylogenetic tree based on 16S rRNA gene sequences showing the relationship among 13 strains and some of their closest phylogenetic relatives



CONCLUSION

The pesticides industries are adding different types of pesticides to environment which bring changes in natural

properties of the soil. Pesticides are observed to influence the physico-chemical and biological properties of soil. The present study has indicated slight variations in observed parameters. The soil samples from five different sites exhibited similar pattern in selected parameters which indicates the changes brought about by deposition from industries. The microbial analysis show diversified bacterial species, mainly dominated by different species of *Bacillus*. As these bacterial species are growing in the pesticides contaminated soil, therefore, this study will help to determine the variations and possibilities for remediation.

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Conflict of Interest: The authors declare that there is no conflict of interest.

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An Analysis on Carboxymethyl Guar Gum/Ag Nanocomposite as a Promising Antimicrobial Agent

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ABSTRACT

Guar gum is one of the naturally-derived polysaccharide, which has provided an alternative to the use of synthetic non-biodegradable polymers which have a deleterious effect on the environment and human health. In the recent past, the use of these naturally derived polymers like guar gum has expatiated in various fields like research, drug delivery etc. In this paper, we have amalgamated one of the derivatives of this polymer, i.e. Carboxymethyl Guar Gum (CMGG), with silver nanoparticles to form CMGG-Silver nanocomposite. We tested this nanocomposite for its antimicrobial activity against different water samples and gram-negative bacteria, *Pseudomonas aeruginosa*. The bacterium, *Pseudomonas aeruginosa*, is involved in many types of skin and blood infections. Also, this bacterium is involved in hospital-acquired infections such as sepsis syndrome and ventilator-associated pneumonia, killing millions of people every year. We have checked the antimicrobial activity by using a varied amount of the nanocomposite. Also, we used two types of nanocomposite solution with varying concentration of CMGG while conducting the experiment. It was observed that a small volume of nanocomposite solution ranging from 500-1000 µL was found suitable for complete inhibition of *Pseudomonas aeruginosa* growth for 96-144 hours. For testing water samples, we collected samples from different locations on our campus. The nanocomposite showed promising results prohibiting the bacterial growth in these samples as well. The above observations can help us to conclude that the CMGG-Silver nanocomposite is a probable candidate for its use as an antimicrobial agent. Future experiments on different bacteria can widen the spectrum of their use in various industrial and biomedical applications.

KEY WORDS: CARBOXYMETHYL GUARGUM, GREEN SYNTHESIS, NANOPARTICLE, NANO-COMPOSITES, PSEUDOMONAS AERUGINOSA.

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INTRODUCTION

In the modern world, humans are looking for different ways to curb dangerous microbes as they have created havoc in the form of diseases. Pathogens have become a major cause of infractions in humans, animals and plants. Microbes are ubiquitous and carry various roles in the environment. Among them pathogenic bacteria have become a major challenge for researchers as many of them have become multidrug resistant. Recent studies show that many commonly occurring microbes such as

Pseudomonas aeruginosa, Staphylococcus aureus and *Streptococcus pyogenes* are associated with hospital acquired infections such as sepsis syndrome and ventilator associated pneumonia, killing millions of people every year. *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* are also responsible for most cases of food poisoning. Nanotechnology is a potent tool to match these antimicrobial compounds that can help in curbing these pathogens as the Nano stuff is dimensionally and structurally similar to most of the biological compounds which makes them one of the promising candidates to be used in biomedical research and other applications such as imaging, gene delivery systems, antimicrobial, food packaging and many more (Waren et al, 1998; Roy et al, 1999; Singh et al, 2008; Mostafa et al, 2018).

Also, antimicrobial packaging is gaining interest and is rapidly expanding with the application of nanotechnology (Sarwar et al 2018). Moreover, the hype in antimicrobial resistance against conventional antibiotics is limiting our medicinal resources at a very fast rate. Therefore, moving on to these Nano-biological materials as antimicrobial compounds can be very fruitful in the coming decades. It will break the dependence over use of synthetic compounds which is widely used in the healthcare sector as well as in the food packaging industry. Among all nanoparticles, silver nanoparticles are toxic for a wide range of microbes which makes them suitable candidates to be used as an antimicrobial agent for different applications. For instance, many of the nanocomposites made from silver are used for food packaging (Liau et al, 1997; Gupta and Silver, 1998; Nomiya et al, 2004; Batista et al, 2019). Carbohydrate silver nanocomposites have been used in antimicrobial activity because of their ability to act as a capping agent and reducing properties due to the presence of carbohydrates (Rao et al, 2012; Xia et al, 2012; Ghobashy et al, 2017) [AG6].

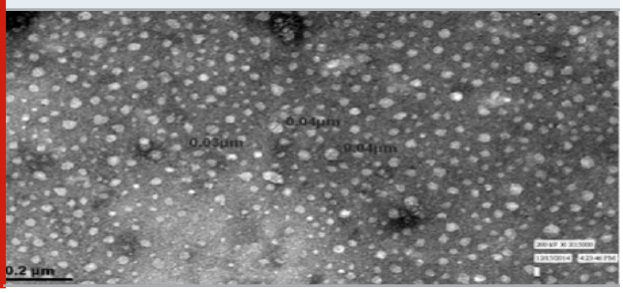
Besides the anti-bacterial nature of silver nanocomposite, biocompatibility, non-toxicity and their ability to assort with natural polymers like Collagen, Gelatin etc. have helped them to be used in tissue engineering and other medical applications like wound dressing (Aktürk et al, 2019). Also, Polymer nanocomposites have been used in water purification (Zheng et al, 2015; Pandey et al, 2017; Mukhopadhyay et al, 2020). On coming in context to the Guar gum/Ag Nanocomposites, a type of Carbohydrate nanocomposite, have also shown excellent antimicrobial activity against *L. monocytogenes, S. typhimurium* etc. and have been used as an active packaging to inhibit the growth of microorganisms (Arfat et al, 2017; Herrera et al, 2018). In an earlier paper, significance of Guar gum and their derivatives as a capping as well as a reducing agent for silver nanoparticles was mentioned (Gupta and Verma, 2014), along with the process of synthesizing Carboxymethyl Guar Gum silver Nanocomposite (CMGG/Ag NC) (Gupta and Verma, 2014). CMGG is one of the anionic semi-synthetic guar gum derivatives (Mukhopadhyay et al, 2020).

This polysaccharide is easily water-soluble. It has a wide range of applications in various industries especially the food industry (Gong et al; 2012; Gupta et al, 2013; Priyadarsini and Biswal, 2020). Lack of data to prove the antimicrobial property of the nanocomposite against different bacteria is one of the reasons we took up this study. This can help in widening the use of this nanocomposite against bacteria like *Pseudomonas aeruginosa*. Testing our nanocomposite against water samples collected from different locations in our college is a step forward to show that this nanocomposite can have multiple areas of application. It helps us in rationalizing that the CMGG-Silver nanocomposite can be used as an alternative to chlorination for water purification as the process of chlorination can itself lead to the generation of certain harmful compounds.

MATERIAL AND METHODS

Silver nitrate ($AgNO_3$) was purchased from Hi Media Laboratories Pvt. Ltd. Carboxymethyl guar gum was brought from Hariom gum Industry, Gujarat, India. All aqueous solutions, used in the experimental work, made using doubled distilled (DD) water. The microbial culture of Pseudomonas aeruginosa was ordered from the National Chemical Laboratory (NCL), Pune, India in the form of slant culture. For testing the use of the nanocomposite in water purification, different water samples were collected within the Manav Rachna International Institute of Research and Studies Campus, Sector-43, Faridabad, Haryana-121004, India.

Figure 1: TEM images of CMGG/Ag NC solution 200 nm scale.



The synthesis of CMGG/Ag NC was done as reported in the literature (Gupta and Verma, 2014). CMGG powder was dissolved in 10 ml double-distilled water (DD) under constant stirring on the magnetic stirrer. After complete dissolution of CMGG in water, the temperature was raised to 90°C. Then, 10 ml of silver nitrate solution was added to the same. The solution was continuously stirred up for 100 minutes after addition. Slowly the color of mixture started changing its color. After some times the color of the solution was converted to brownish color. This confirmed the formation of the nanocomposite. For the characterization, CMGG/Ag NC morphology was characterized using Transmission Electron Microscope (Tecnai, G2- 200 KV HRTEM, SEI company, Holland).

RESULTS AND DISCUSSION

For the morphological study, CMGG/Ag NC morphological study was done using TEM images. TEM images shown in figure 1. The presence of spherical shapes in image confirmed formation of nanocomposites.

Antimicrobial activity of CMGG/Ag NC solution against *Pseudomonas aeruginosa*: Antimicrobial activity of CMGG/Ag NC was tested using the spread plate method. A broth culture was prepared for Pseudomonas aeruginosa. It was kept at 370 C in a sterile environment for 12-14 hours. Nutrient Agar medium was prepared for the growth of *Pseudomonas aeruginosa*. Two CMGG/Ag NC stocks were prepared using different concentrations of CMGG i.e. 1% and 2% (Orsu and Matta, 2020).

The Agar media was plated in seven different Petri plates. Out of these 7 plates, plate 1 was chosen as a control in which no further addition was done. 500µL of the broth culture of log-phase *Pseudomonas aeruginosa* was spread over the plated agar in the remaining six plates. Out of these six plates, different volumes of 1% CMGG/Ag NC was spread on three plates and the 2% CMGG/Ag NC was poured on the other triples. All the plates then incubated at 370 C under sterile conditions. Growth of microbes recorded with time. The observations are tabulated in Table 1.

Our results prove that the CMGG-Silver nanocomposite exhibits antimicrobial property against *Pseudomonas aeruginosa*. These results can also be viewed as an extension to the previous work of Gupta and Verma to test the antimicrobial activity of Carboxymethyl guar gum–silver nanocomposite film against E.coli and B. subtilis (Verma and Gupta, 2015). In our experiments, we used the spread plate method for the first time in order to test antimicrobial activity. The earlier attempt that was made against *E. coli* and *B.subtilis* employed the use of a nanocomposite film (Orsu and Matta, 2020).

Antimicrobial activity of CMGG/Ag NC solution for different water samples: The collected water samples were tested for the action of CMGG/Ag NC using the spread plate technique. Instead of using the bacterial culture, water samples were spread on Nutrient Agar plates and later on the CMGG/Ag NC solution was added. This time we used a standard 1 % concentration of CMGG in the nanocomposite stock solution. The plates with nanocomposite were compared against the plates containing only water samples. The growth of microbes with time is reported in Table 2 with appropriate figures (Orsu and Matta, 2020).

Note: The figures reported were observed after 24 hours of incubation.

Table 1. Anti-microbial activity against Pseudomonas aeruginosa

S.no.	CMGG conc. (%)	CMGG/AgNC (µL)	24 hrs	72 hrs	96 hrs	144 hrs	Figure no.
1	1	500	no	no	no	**	2(a)
2	1	750	no	*3	*3	**	2(b)
3	1	1000	no	**	**	**	2(c)
4	2	500	no	*2	*6	*6	2(e)
5	2	750	no	*1	**	**	2(f)
6	2	1000	no	no	no	no	2(g)
7			no				2(d)

Figure 2. (a to g): The antimicrobial activity of nanocomposite against *Pseudomonas aeruginosa*

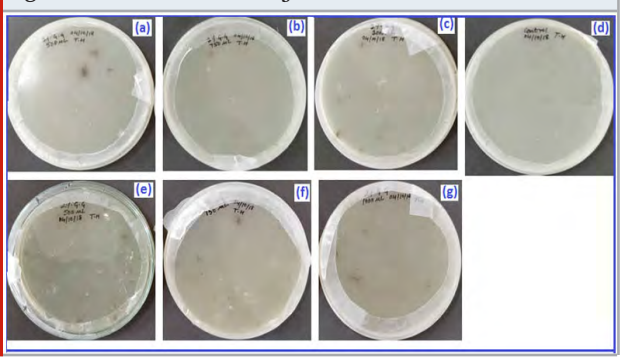
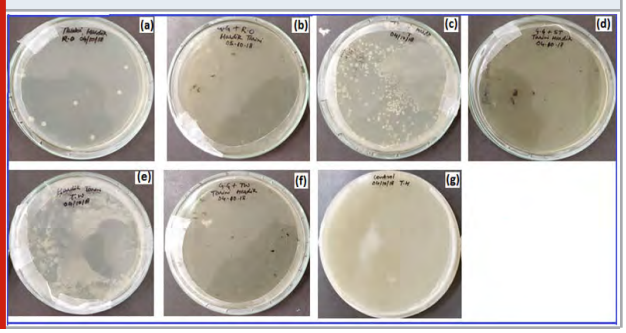


Figure 3. (a to g):Antimicrobial activity of CMGG/Ag NC solution for different water samples (references given in table 2)



The results demonstrate that this nanocomposite can have multiple areas of application. It helps us in rationalizing that the CMGG-Silver nanocomposite can be used as an alternative to chlorination for water purification as the process of chlorination can itself lead to the generation of certain harmful compounds. A recent attempt has been made to use this nanocomposite for wound healing. These types of nanocomposites can also be used in the process of drug delivery but meagre information is available on this. Another potential field of application is their use as a replacement for antibiotics which can be validated by conducting in vivo studies thereby playing an important role in curbing Antimicrobial Resistance (Orsu and Matta, 2020).

Table 2. Water sample analysis (RO: Reverse Osmosis Water, TW: Tap Water, ST: Septic Tank water Vs GG: CMGG/Ag NC solution)

S.no.	Sample	CMGG conc. (%)	CMGG/Ag NC (µL)	24 hrs	72 hrs	96 hrs	144 hrs	Figure no.
1	RO	1	500	*	*13	**	**	3(a)
2	RO + CMGG Ag NC	1	500	no	no	no	*12	3(b)
3	ST	1	500	*	**	**	**	3(c)
4	ST + CMGG Ag NC	1	500	no	no	no	*1(big)	3(d)
5	TW	1	500	**	*	**	**	3(e)
6	TW + CMGG Ag NC	1	500	no	*6	**	**	3(f)
7	Control			no				3(g)

*n refers to number of colonies, ** refers to multiple colonies. RO: Reverse Osmosis Water ,TW: Tap Water, ST: Septic Tank water , GG: CMGG/Ag NC solution

CONCLUSION

Antimicrobial activities of CMGG/Ag NC were tested against Pseudomonas aeruginosa as well as the microbial population in different water samples. Comparative study of the growth of bacteria with time has revealed the effect of CMGG/Ag NC on microbes. It was observed that a small quantity (500µL) of nanocomposite was sufficient for complete inhibition of bacterial growth up to 96 hours and a small increase in the volume of nanocomposites (up to 1 mL) was found suitable for complete inhibition of Pseudomonas aeruginosa growth up to 144 hr. From the results, it can be concluded that CMGG/Ag nanocomposites may be used as an antimicrobial agent for different purposes especially in water purification. Further research on its intake in the human body and the spectrum of its antimicrobial infection can help us to link this to in vivo treatment of bacterial infections.

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Conflict of Interest: The authors declare no conflict of interest.

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The Effect of Using Artificial Intelligence on the Quality of Decision-Making in Various Organizations: A Critical Survey Study

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ABSTRACT

The present study aimed at identifying the status of previous studies related to the subject of the impact of artificial intelligence on the quality of decision-making in organizations, and providing a critical review of it. In fact, this study attempted to fill several gaps related to the recent studies in this field, which are considered few from the researchers' point of view. Moreover, it dealt with the most important findings and recommendations reached by those studies in relation to the impact of the use of artificial intelligence on the quality of decision-making in various types of organizations, the types of decisions that can be supported by artificial intelligence techniques. in addition, it identified the most important algorithms, methods, techniques and models of artificial intelligence revealed by these studies that could help providing an accurate decision-making. In order to achieve these goals, this study used the descriptive, analytical, documentary approach. Its results showed that the use of artificial intelligence techniques positively affects the accuracy and quality of decision-making in different forms of these organizations no matter what structure of these organizations is. The study also found that the most important popular techniques and algorithms of artificial intelligence that can be used to improve the quality of decision-making are Support Vector Machine, Artificial Neural Networks, Back-propagation neural networks, Bayesian networks, Adaptive networks, Fuzzy inference system, Random Forest, Decision Tree, Logistic Regression, and K-Nearest Neighbor. The study recommended the necessity of conducting more experimental and exploratory studies on the impact of applying artificial intelligence on the quality of decision-making within organizations. Furthermore, it recommended designing models and action plans regarding employing artificial intelligence in decision-making in order to facilitate their implementation use, understanding, and the analysis of their results.

KEY WORDS: ARTIFICIAL INTELLIGENCE (AI), INFORMATION TECHNOLOGIES, DECISION MAKING, QUALITY OF DECISIONS, PERFORMANCE OF ORGANIZATIONS, FOURTH INDUSTRIAL REVOLUTION.

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INTRODUCTION

The decision-making process in organizations is one of the most difficult stages in the administrative processes. Indeed, regardless to the leaders or to the decision-maker's analytical capabilities, intelligence, and experiences, there is always a possibility of making wrong decisions. There is no doubt that the tremendous development in recent years in the field of AI, which in turn has brought

about a qualitative leap in various fields and disciplines - especially in the areas of various organizations management - has greatly helped in facilitating and improving the quality of various administrative activities in organizations. Eletter, Yaseen, and Elrefae (2020) have clarified, through a study which they conducted on a banking organization, that the intelligent information system that relies on AI would provide organizations' decision-makers with value-added information that helps them reduce uncertainty about the decision outcome and enhance the quality of banking services, which gives them a competitive advantage and better performance.

Recently, Bosco (2020) have showed that AI has a role in raising the efficiency of organizations 'performance in terms of employing artificial intelligence to make better and accurate decisions in order to achieve the organization's goals. Este et al., (2020) have mentioned that the use of AI has become very popular now, and stated that it is an amalgamation of several technologies, and that it is the science and engineering of the intelligent machines manufacturing. Vedamuthu (2020) clarified what is meant by AI techniques stating that they are those techniques concerned with creating computer systems based on simulating the way humans use their senses, intelligence and abilities to accomplish the tasks that only the human mind could accomplish.In fact, artificial intelligence has begun to be introduced in healthcare for purposes such as facilitating clinical ordering systems, and identifying patients at high risk for screening tests. Besides that, artificial intelligence will have an increasing impact in healthcare and other disciplines and fields, because AI is superior in some aspects to human intelligence such as visuospatial processing speed and pattern recognition (Este et al., 2020).

Where, millions of information data are stored inside the computer to form a main database in analogy to the information stored inside the human mind, through learning and acquired daily experiences. Then, special programs are developed to enable computers to deal with this information in a logical manner, so these computers can solve the problems and make the decisions (Jabbari, 2016). Beside the previously mentioned about the importance of artificial intelligence, the idea of preparing this scientific study came with the aim of reviewing previous studies related to the topic to identify the extent of the impact of recent developments in AI during the past years on quality, efficiency and accuracy of decision-making in organizations. This scientific study consists of seven parts namely introduction, study problem, study methodology, previous studies, results, analysis of results, and conclusion and recommendations.

Research problem: The recent years have witnessed rapid technological developments and radical changes through the emergence of many modern technologies for information systems, most notably AI techniques. Vedomuthu (2020) indicated that AI has become a fundamental topic in the management of organizations due to its capabilities that allow cooperation between the organization staff and AI in order to improve the decision-making process. As the process of making the right decision in organizations is the basis for their success and for achieving their goals, we find that most organizations have resorted to the use of AI in decision-making processes to make use of their capabilities in analyzing data and deconstructing problems to come up with the best and most accurate decisions. Based on the above, the researchers considered the necessity to prepare a scientific paper aiming at identifying the extent to which the use of AI in various organizations affects the quality of decision-making. Thus, the study problem can be stated through the following basic question: What is the effect of using AI on the quality of decision-making within all types of organizations? In addition, the study seeks to answer the following sub-questions, what kinds of decisions can be supported by AI techniques? what are the most important algorithms, methods, techniques and models of AI found by the study that can help in accurate decision-making?

METHODOLOGY

This study will use the descriptive analytical documentary approach, which is based on reviewing documents and literature such as scientific researches, articles, books, etc., and then studying, describing, and analyzing them in details in order to extract conclusions and indications related to answering the study questions (Al-Assaf, 2006). Therefore, this study will analyze and evaluate the published studies on the impact of AI on the quality of decision-making in the organizations. Knowing, these studies were reached via databases available through the Saudi Digital Library and the search engine Google Scholar as research tools.This section aims at looking over and critically reviewing a number of studies related to the topic of AI and its impact on the quality of decision-making in organizations, and will be arranged in descending chronological order from the most recent to the oldest. The studies fall within the period of time 2010-2020 AD. The total number of the reviewed studies is (12) which are distributed as follows:

Stone et al (2020) worked on "Artificial intelligence (AI) in Strategic Marketing Decision-Making: a Research Agenda" which described identifying the effect of AI on the efficiency of marketing decisions through a methodology based on reviewing the literature on AI applications in strategic situations. Some marketing experts has been also invited to contribute with their views to this research. The results indicate that there is very little research on the applications of AI on the strategic marketing decisions. Therefore, the importance of this study appears in it is one of the few studies that dealt with this field. This study showed that research in the field of AI linked to strategic marketing decisions seems challenging due to several restrictions and reasons namely the fact that it is a commercial field that contains sensitive data for organizations. Besides, companies that have had successful experiences in using AI in the field of decision-making do not want to spread its experience to prevent its competitors from getting benefit from it.

The work of Vedamuthu, (2020) entitled as "Artificial Intelligence and Human Collaboration in Project Decision-Making", aimed at knowing the possibility of applying cooperation between humans and AI in project management to determine the current uses, and to create an applicable decision model for deciding (when and how to apply AI in project management). And the mode is designed to enhance quantitative decisions in the strategic decision-making, in order to reduce bias, human error and uncertainty in decisions. The researcher indicated that there is currently no decision model to determine how and when to cooperate with AI to make strategic project decisions. Therefore, this study came to cover this gap existing in previous studies, by developing a decision model based on flowcharts to facilitate the use of AI in strategic decisions. These charts showed how and when AI can be used in project decision-making and use this information to support the use of the decision model. The researcher reached these charts through a literary review of previous studies. Therefore, the researchers of this study believe that this model or proposal still needs more field studies to demonstrate its feasibility and effectiveness.

Bejger and Elster (2020) described "Artificial Intelligence in Economic Decision-Making: How to Assure a Trust" which deals with the decisions made by modern AI models, which are called "black boxes", which are systems that use statistical algorithms and do not reveal their internal mechanisms (such as the neural Artificial network). Thus, people do not trust them in decision-making. These advanced models came after the glass-box models, which are characterized by transparency and the use of symbolic results that are understandable to humans, but are less sophisticated than black box models. The research showed that AI models need regulations and laws for work. It also need to convert these regulations and laws into action plans for decision-making practices, and at the same time, need technical and practical tools to ensure confidence and transparency in decisions.

The research has listed examples of initiatives from different organizations and countries such as Poland, Britain, U.S.A, Canada and Japan in order to make AI models more transparent and reliable. In this regard, the research suggested conducting fundamental adjustments to one of the practical tools, which is the famous standard CRISP DM process model - which is considered one of the practical tools for machine learning and data mining - in order to enhance its work transparency. The research also explained one of the official technical tools, which is the "Anchors" tool, which can support AI models to make them more accurate, reliable and transparent. The strength of the research lies in all the solutions and tools that it presented, and the multiple methodologies it used in conducting the study namely inductive and deductive inference methods, descriptive and comparative analysis, and the experimental method, (Beiger 2020).

In the study of How et al (2020) entitled "Artificial Intelligence-Enhanced Decision Support for Informing Global Sustainable Development: A Human-Centric AI-Thinking Approach" the researcher dealt with AI and its ability to inform sustainable development and its associated organizations through assisting in analyzing primary social and environmental data in order to support decision-makers to take appropriate and correct action on sustainability in development. The paper also made clear that the use of AI was not easy for those who are not trained and who are not proficient in the computer using.

The strength of this scientific paper lies in its clarification of the method of using AI through the researcher's use of a "human-centric probabilistic reasoning approach", which is the Bayesian Network (BN). That technology was benefited people, who did not have talent and experience in using computers, in using of the AI to analyze the environmental performance indicators (EPI) data related to sustainability. Moreover, this study is also strong in its application of the study methodology over (180) countries, which resulted in important conclusions such as the fact that the BN approach can simulate many scenarios that are not available in the real world. This helps officials and organizations in accurate decision-making and predictive analysis, especially under the conditions of best and worst results based on EPI indicators at the global system level. The results of the study found that there are four of the EPI indicators, which are the most influential in all scenarios. These are as follows, classified from best to worst, drinking water quality, sterilization, exposure to lead, and wastewater treatment, (How et al 2020).

"A Study on Artificial Intelligence Interaction with Organizational Performance" aimed at bridging the gap related to implementing AI in organizations in order to raise performance, facilitate decision-making and raise efficiency and quality. This study used the descriptive approach based on electronic questionnaires and interviews on a sample of (100) employees in order to know the impact of AI on raising the efficiency of the performance of organizations and the possibility of employing AI in organizations to make accurate decisions, in order to achieve the organization goals. The results found that organizations are ready to employ AI in several areas within the organization in order to make decisions, raise efficiency, reduce costs, and increase profits. However, there are obstacles to applying AI techniques namely cyber security challenges, protecting customer data, and insufficient budgets, (Bosco,2020).

Lange, Konig, & Busch, (2020) worked on "Changing the Means of Managerial Work: Effects of Automated Decision Support Systems on Personnel Selection Tasks" the researcher touched on ways to enhance the quality and efficiency of decision-making through the use of automation based on AI, and to know its impact in support of administrative tasks and decisions related to employee selection. The strength of this study lies in the fact that it came in contradiction to most theoretical studies that dealt with these topics. It was distinguished by the abundance of its hypotheses subject to the experimental laboratory study to ensure the accuracy of the results,

which amount to six hypotheses, and applying it to a random sample of three groups of specialists in personnel affairs. The sample was subjected to five repeated rounds of personnel or employee selection processes. The results showed that satisfaction with employment decisions was higher for the participating group that received support from the automated system after the group had previously performed a human-processing process for applicants. Moreover, participants in this group showed a sharp increase in self-efficacy in employee selection compared to the other groups.

The recent work of Pourhomayoun Shakibi (2020) entitled "Predicting Mortality Risk in Patients with COVID-19 Using Artificial Intelligence to Help Medical Decision-Making in the wake of the COVID-19 pandemic" designed and developed a predictive model that relies on AI and machine learning algorithms to identify health risks and predict mortality risk among COVID-19 patients. The study used documented and laboratory-confirmed data of 117,000 patients all over the world with COVID-19. The study proposes an AI model to help hospitals and medical facilities for identifying who needs attention first, who has a higher priority for hospitalization, patients sorting and ranking when the system is very crowded, and eliminate delays in providing necessary care. The results showed an overall accuracy of 93% in predicting the death rate. Furthermore, the study used several machine-learning algorithms to predict the death rate in patients with COVID-19. The strength of this study lies in its practical methodology based on a large and reliable sample, as well as in its proposed model, which has proven its effectiveness.

Chernov et al (2019) explained "The Usage of Artificial Intelligence in Strategic Decision Making in Terms of Fourth Industrial Revolution"finding out the effect of using AI on the accuracy and quality of strategic decision-making through primary and secondary research work methods. In the first stage of secondary research, the researchers collected huge data from independent studies, technical journals, and reliable data sources. Next, the results of the first phase were the basis for the authors 'work in the second phase of the study. In the initial research, the authors used the Delphi method to collect data by interviewing several experts and influencers in the field about their use of AI to make strategic decisions. However, we believe that one of the weaknesses of this study is that the type and number of the sample were not explained. The results of the study showed that, only, 24% of managers of organizations are willing to use AI applications in order to support the efficiency of strategic decision-making. Most of these managers were from technical or banking companies, which justified their knowledge of the capabilities of AI, while the rest of the managers were found to have insufficient background on the capabilities of AI to support decision-making, especially the strategic ones.

More (2019) worked on "Disaster Management Using Artificial Intelligence" focused on the use of AI in managing crises and disasters and making decisions in times of crises that lead to great damage to the society and economic crises. In this study, AI can be used to analyze data that can be used to predict a future event, create awareness, and then make the decision for the situation. The results of the study questionnaires showed that 95% of the participants feel that AI can be used to manage crises and 80% of them feel that the impact of AI improves their lives for the better. On the other hand, we believe that the fundamental problem of this research is that the study was applied to a sample of less than 100 people, which prevents a conclusive generalization of its results. Hence, the study needs to be re-applied to a larger sample.

The exploratory study of Schmidt (2019) titled as "The Impact of Artificial Intelligence on Decision-Making in Venture Capital Firms" examined the employment of AI in the decision-making process in venture capital. Venture investors have to make decisions under uncertainty, time pressure and suffering from self-decisions. This study examines the potential of AI to overcome these challenges and improve the process. The results are based on a qualitative analysis based on 12 interviews with venture investors, AI experts, and companies providing venture capital solutions, as well as secondary data in the form of academic articles and online journals. The results reveal things. Fist, the AI is actually implemented and the interviews show that AI can be implemented at every step of the decision-making process, especially at the beginning of the decision-making process. Second, the use of AI improves the decision-making process by reducing uncertainty and bias and increasing productivity and efficiency. Third, the results determine use cases, implementation challenges and usage implications. Last, the results also show that the venture investors were able to make more accurate and better decisions using AI, which positively affected their portfolios.

Earlier, Liao et al (2013) published a work, "Applying Artificial Intelligence Technology to Support Decision-Making In Nursing: A case study in Taiwan" which aimed at identifying the effect of using an AI system on the ability to make decisions, solve problems and diagnose patients, on a sample consisting of Nurses inside a Taiwanese hospital. After the system implementation, the results were that the accuracy and efficiency of decision-making and problem solving were of a higher degree and in a less time, compared to the conditions before the application of the artificial system. However, the compatibility rate between the nurses' diagnosis and the system diagnosis was only 87%. It is worth mentioning, this study used an AI system based on back-propagation neural networks, data mining tools, and statistical analysis.

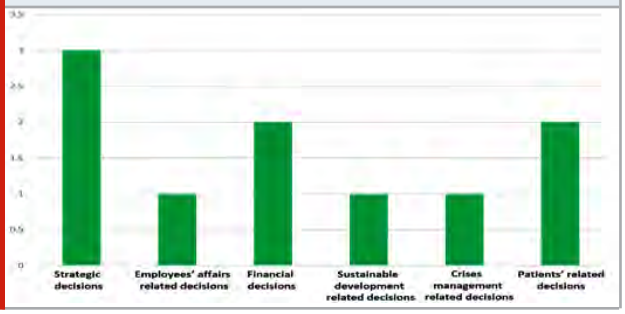
Similarly Eletter et al (2010) through their "Neuro-Based Artificial Intelligence Model for Loan Decisions" description, aimed at designing a tool to support decisions related to classifying loan applications submitted by clients into good or bad in Jordanian commercial banks. This study developed a proposed model that uses an

artificial neural network as an enabler to evaluate loan and credit applications. The model used a multi-layered neural network containing a back-propagation learning algorithm to build the proposed model. This network, by its adaptive nature, can implement the new data without reprocessing the old data. After the experimental application of the proposed model, the results indicated that artificial neural networks are a successful technology that can be used to efficiently evaluate loan applications and make sensitive decisions related to them. It is worth noting that researchers have had difficulty identifying the variables that affect approval of the loan decision.

RESULTS AND DISCUSSION

Through reviewing of previous studies, the researchers reached many aspects and results, the most important of which are: Previous studies have shown the possibility of using AI in different types of decisions. We found that some studies have used AI on strategic decisions such as the work of (Stone et al. ,2020), (Vedamuth, 2020), (Chernov, Chernova, & Komarova,2019). Other studies have benefited from it in decisions related to sustainable development like study (How, et al 2020) as well as the possibility of using it on financial decisions such as: (Schmidt, 2019) and (Eletter et al 2020). In addition to decisions related to patients such as the work of Liao, Hsu, Chu, (2013) and (Pourhomayoun, Shakibi, 2020). Moreover, some studies have shown the possibility of using it in employees' or personnel affairs related decisions such as study (Langer, Konigm Busch, 2020). As for the work of More, (2019) it clarified the possibility of using AI in decisions made in times of disasters and crisis as illustrated in the following figure(1):

Figure 1: Types of decisions in current study



Some previous studies have showed some difficulties that may face organizations during the applications of AI in decision-making processes. For instance, (Stone et al, 2020) found difficulty in the field of AI related to strategic marketing decisions for several reasons including the fact that it is a commercial field containing sensitive data for organizations, also companies that have had successful experiences do not want to share their experiences with others so that competitors will not benefit from them. Bejger amd Elster, (2020) found that AI models still need some regulations and laws, and then convert them into action plans for decision-making practices, and there is still a need for more technical and practical tools to ensure confidence and transparency in the decision. While How et al, (2020) have shown the difficulty of using AI lies in the inability of untrained employees, who are unable using the computer to deal with AI techniques. Bosco, (2020) also showed that the most important difficulties and challenges facing the use of AI in decision-making processes are challenges related to cybersecurity, customer data protection, and insufficient budgets. As for the work of (Chernov et al, 2019), the obstacle is the lack of knowledge of some managers of the capacities and capabilities of AI to support taking accurate decisions.

Eletter et al (2020) faced the difficulty of identifying the variables that affect strategic decisions regarding loans.

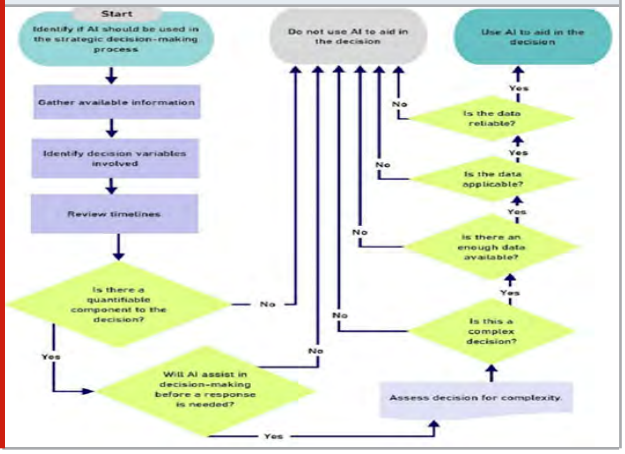
The work of Schmidt, (2019) is considered one of the important studies as it focused on the importance of employing AI in the decision-making process in venture capital. Thus, risk investors have to make decisions in light of uncertainty, time pressure and suffering from self-decisions. The study found that AI is actually used in every step of the decision-making process, especially at the beginning, and that the use of AI improves the process of decisions making by reducing productivity and efficiency. The study also showed that the risk investors were able to make more accurate and better decisions using AI, so this positively affected their portfolios. uncertainty and bias and increasing.

Some previous studies have suggested models, methods, algorithms and types of AI to aid in the decision-making process, including: Bejger and Elster, (2020) proposed fundamental modifications to the famous standard CRISP DM process model - which is considered one of the practical tools for machine learning and data mining - in order to enhance the transparency of its work, by enriching it with several regulations, laws and guidelines. As well, the study suggested technical and formal tools to enhance confidence and transparency in AI models including the "Anchors" method or tool that can support AI models to make it more accurate, reliable and transparent, especially with regard to economic decisions.

As for study of How et al (2020), it illustrated a method for using artificial intelligence. Where the researcher used a "human-centric probabilistic reasoning approach", which is the Bayesian Network (BN). This has benefited people who do not have the talent and the experience of using computers in their harnessing of AI to analyze environmental performance indicators (EPI) data related to sustainability. Pourhomayoun and Shakibi, (2020) designed and developed a predictive model relies on AI and machine learning algorithms and techniques including Support Vector Machine, Artificial Neural Networks, Random Forest, Decision Tree, Logistic Regression, and K-Nearest Neighbor. All that, in order to develop an AI model to help hospitals and medical facilities identify who needs attention first, sort patients when the system is overcrowded, and eliminate delays in providing needed care.

Recently Eletter et al, (2020) have designed a tool to support decisions related to classifying loan applications submitted by clients into good or bad in Jordanian commercial banks by developing a proposed model that uses the artificial neural network- the MLFF network- as an enabler to evaluate loan and credit applications. A multi-layered neural network was used to build the proposed model, and this network, by its adaptive nature, can implement the new data without reprocessing the old data. Liao et al, (2013) used an AI system based on neural networks and compared the results of the pre- and post-experience of the studied system that used fuzzy inference system, which based on an adaptive network and back-propagation neural network. Both Eletter et al (2020) and Liao et al (2013) have used machine-learning algorithms based on back-propagation neural networks and adaptive networks, whereas Vedamuthu, (2020) developed a decision model based on flowcharts with the aim of codifying the process of using AI in strategic decisions. It can be illustrated as follows in figure (2):

Figure 2: The process of using AI in strategic decisions and the flowchart (Vedamuthu, 2020)



The work of Schmidt, (2019) is considered one of the important studies as it focused on the importance of employing AI in the decision-making process in venture capital. Thus, risk investors have to make decisions in light of uncertainty, time pressure and suffering from self-decisions. The study found that AI is actually used in every step of the decision-making process, especially at the beginning, and that the use of AI improves the process of decisions making by reducing uncertainty and bias and increasing productivity and efficiency. The study also showed that the risk investors were able to make more accurate and better decisions using AI, so this positively affected their portfolios.

Langer et al (2020) depicted the effect of using AI in supporting decisions related to personnel affairs, was unique among the rest of the studies by the abundance of its hypotheses subject to the experimental laboratory study to ensure the accuracy of the results, which amount to six hypotheses. The researchers applied this study to a random sample of three Groups of personnel specialists who were subjected to five rounds of personnel selection processes. The first group received an evaluation of job applicants with the help of an automated support system before the participants processed the applicants' information personally (pre-processing support group). The second group obtained an evaluation of the applicants with the help of an automated support system after they processed the applicant information personally (post-processing support group). The third group did not receive any automatic support in the assessment (no support group).

The results showed that satisfaction with the decision was higher for the post-treatment support group, i.e. the second group. Moreover, participants in this group showed a sharp increase in self-efficacy in employee selection compared to the other groups. However, the researchers of current study see that the weakness of this study is in the fact that this experiment was not completely real, but rather a simulation, as the participants were not managers but rather people interested in the field of personnel affairs, and that the applicants 'applications for employment and employment processes were not real.

The paper of Pourhomayoun and Shakibi, (2020) is the only study that used a technical tool to verify the efficiency and effectiveness of the proposed AI model in study, the tool is the Confusion matrix. Bosco, (2020) agreed with study of Schmidt (2019) that the use of AI in the decision-making processes of organizations helps in facilitating and improving the accuracy of decision-making and reducing uncertainty, as it contributes to raising efficiency, increasing productivity and profits, and reducing costs. The study of Chernov et al, (2019) was distinguished by containing important statistics, as shown in the next figures, (figure3 and 4):

Figures (3 and 4), from study (Chernov et al, 2019, 24), show the statistics on the answers of the study participants

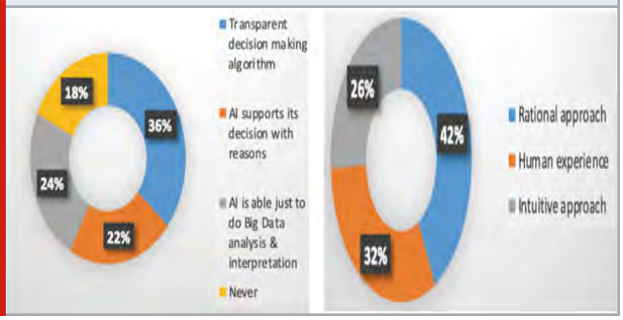


Figure 3 illustrates managers' responses regarding the conditions under which they are willing to transfer strategic decision-making to AI. Where 22% of them refused to use AI -categorically-in all circumstances, maybe because they do not believe in AI abilities. As for the rest, which is 78%, they agreed to use artificial intelligence in only three cases, namely: If AI technology has algorithms that help in the transparency of decisions, if the AI technology able to provide reasons to the decisions or in the case of big data analysis and

interpretation. So, the previous study considered this approach to be the correct approach -to some extent- for many reasons, because the strategic decision-making process within artificial intelligence depends - only - on a rational approach, but skills such as abstract thinking, intuition and context analysis are not available for the smart machine. Where abstract thinking helps a person to work on different concepts basis. Context analysis helps the human to make strategic decisions when he has a lack of information or in conditions of asymmetry of this information. Intuition is the ability to make decisions without thinking logically or using logic, but using a combination of emotions, feelings and past experience.

According to Figure 4, 26% of managers use intuitive methods, 42% of them use rational methods, and 32% of them use personal experiences, in the strategic decision-making process. Thus, combining both approaches - rational and intuitive - appears to be the most effective method of strategic decision-making. Where with such a combined method, the intuitive decision is verified by a rational approach, and is adjusted if necessary. Therefore, the most effective method for the strategic decision-making process- based on the previous study- can be considered the cooperation of man and artificial intelligence, so that the person uses an intuitive approach to make a strategic decision, and then uses the rational approach that uses artificial intelligence. The results of the study have showed the following: The previous studies have been applied to various types of organizations, such as administrative, financial, health, and environmental organizations etc. Most of the previous research has shown that the use of AI techniques positively affects the accuracy and the quality of decision-making in the various types of these organizations.

1. The studies showed the possibility of using AI in different types of decisions, such as financial, strategic, personnel, patients-related, disaster management and sustainable development decisions.
2. The most important and well-known mentioned techniques and algorithms of AI that can be used to improve the quality of decision-making are Support Vector Machine, artificial neural networks, back-propagation neural networks, Adaptive Network, Bayesian Network, fuzzy inference system, Random Forest, Decision Tree, Logistic Regression, and K-Nearest Neighbor.
3. Based on study (Vedamuthm 2020), it is possible to use AI applications in decision-making processes when the following conditions are met:
4. The presence of complex decisions, adequate reliable applicable data, quantifiable or measurable elements within decisions, and when the decision support is needed before the response is needed.
5. There are technical tools to verify the efficiency and effectiveness of the proposed AI models such as the Confusion matrix.
6. The researchers noticed - through a review of previous studies - several gaps. First, the related studies concerned with researching the subject of this study, which is the effect of applying AI techniques on the quality of the decision, are considered very few. Second, some previous studies were not clear in their methodology. Some of them did not adopt large and sufficient samples during the experiment, or did not explain the nature and characteristics of these samples such as (Vedamuthu,2020), (More, 2019), (Bosco, 2020), (Schmidt, 2019), (Liao et al,2013) (Eletter et al, 2020) and (Chernov et al, 2019)

All previous studies agree with the current study in that they all aim at knowing the effect of applying AI techniques on the quality of decision-making. The current study differs from previous studies in that it seeks to know the effect of applying AI on the quality of decision-making for different types and forms of organizations and decisions, and therefore it is not restricted to one type of organizations or decisions like the previous studies. The current study also differs in that it makes an inventory of the most important technologies, algorithms and models of AI that contribute in efficient and high-quality decision-making within organizations.

CONCLUSION AND RECOMMENDATIONS

Depending on the above findings, the researchers reached the following recommendations:-The necessity of designing models and action plans regarding employing AI in decision-making, in order to facilitate implementation and work on them, and to facilitate understanding and analysis of their results.- The need to conduct more experimental and exploratory studies on the impact of applying AI on the quality of decision-making within organizations, especially in the Arab countries. Because the researchers found that, these studies within the Arab geographical area are rare.- The necessity of conducting experimental studies regarding employing AI in decision-making. The same applies to other forms of institutions, organizations - and the decisions related to them - that have not been considered in these studies like agricultural, educational, and commercial organizations, customer service centers and factories etc.- The necessity to hold local and international conferences and seminars in sufficient and periodic manner in order to introduce the novelties and developments in this field.

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The Durum Wheat Gene Sequence Response Assessment of *Triticum durum* for Dehydration Situations Utilizing Different Indicators of Water Deficiency

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ABSTRACT
In the following research, forty durum wheat gene sequences (Triticum durum) were evaluated in water tensioned and also enough-watered conditions in three years 2015 till 2018 product years. In every surrounding, the gene sequences were estimated utilizing the whole block models that are chosen accidentally by 3 iterations. From the data of the seed product, dehydration endurance indicators including STI, SSI, GMP, MP, TOL, YSI and YI have been measured for each gene sequence. The yielding has investigated as attained from a whole block model that is chosen accidentally. Considerable variations between gene sequences have been recognized for the indicators of the whole dehydration endurance. Large product amount in tension was presented by gene sequence 'Genotype NO.40 and non-tension surroundings was presented by and 'Genotype NO.32'. The highest amount of STI, MP and GMP indicators have related to gene sequence 'Genotype NO.35'. The largest amount of YI has been from gene sequence 'Genotype NO.39' and 'Genotype NO.21'. Association ratios showed that TOL, MP, GMP, STI, HM, and YI indicators can efficiently be utilized to screen the dehydration endurance gene sequence. Utilizing MP, GMP, TOL, YI and STI indicators, gene sequence UPGMA assortment has been accomplished and 3 groups have been initiated where matched the bi-plot investigation outcomes. in this investigation, by considering the outcomes, Genotype NO.10 and Genotype NO.35 was the maximum dehydration endurance gene sequence that was classified as cluster A. endurance indicators containing STI, GMP, and MP are appropriate for wheat dehydration endurance gene sequence choice has been proposed.

KEY WORDS: BI-PLOT, MULTI-VARIABLE INVESTIGATION, WATER DEFICIT, TRITICUM DURUM DESF, PRODUCT DURABILITY.

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INTRODUCTION

In the current time, Nouri and co-workers in 2011 have represented that durum wheat has been raised often in the Mediterranean rainy regions under the situations that are full of tension and unsteady environment (Gholamin and Khayatnezhad, 2020a). Leilah and co-workers in 2005 have illustrated that producing the high production cultivars of the wheat under dehydration situations in dry and semi-dry areas is a significant

purpose of breed programs. Giunta et al (1993); Simane et al (1993); Gholamin and Khayatnezhad (2020b) Abayomi and Wright (1999) have represented that the tension of dehydration could decrease the whole product ingredients, however especially the number of clusters that are productive per unit region and the number of seeds per cluster (Gholamin and Khayatnezhad 2012, Khayatnezhad and Gholamin 2012, Gholamin and Khayatnezhad 2020, Gholamin and Khayatnezhad 2020).

While Chmielewski and Kohn in 2000 have demonstrated that high temperatures and dehydration throughout ripe have negatively affected the weight of grain. Generation of wheat in the Mediterranean area is mostly restricted by sub-optimum humidity situations. Khayatnezhad et al., 2020 have represented that signs of herb disposal that are able to see for dehydration in the vegetation condition are leafage wilt and a reduction in the height of herb, herbs number, and leafage space, and lag in the seeding and flower precision. Also, Li et al., in 2000 have demonstrated that the divergence of genes associated with environmental variations has been observed for emmer wheat. Reddy and co-workers in 2004 and Zhao and co-workers in 2008 have shown that comprehension of herb responds for dehydration and also the main section of producing production water deficit have high attention.

Mohammadi et al., in 2010 have demonstrated that the gene sequence corresponding product function in dehydration tensioned and desirable surroundings beseem to be an ordinary beginning step in the description of favorable gene sequence for random rain situations. Betran et al., in 2003 and some investigators have believed in choice under desirable situations, Rathjen in1994 and other ones are believed in a purpose tension situations. While Byrne and co-workers in 1995 and Rajaram and van Ginkel in 2001 still have selected a middle-point and have believed in choice under tension and desirable situations (Khayatnezhad 2012, Khayatnezhad and Gholamin 2012, Khayatnezhad and Gholamin 2020).

In general, various approaches have been suggested for the corresponding dehydration endurance and resisting gene sequence choice, while Fisher and Maurer in 1978 stated that the product of achene in dehydration surrounding can be regarded as a dehydration endurance indicator. And Blum in 1988 suggested which the choice of gene sequence for desiccation endurance should be related to choice for the more production in the non-tension surroundings. Therefore, with estimating of gene sequence product in dehydration and good-watered surroundings, we can choose endurance gene sequence for dehydration. Fernández in 1992 has proved that there can be some choice indices to screen dehydration endurance gene sequence like GMP and STI, Rosielle and Hamblin in 1981 have studied MP and TOL, Jafari and co-workers in 2009 have studied harmonic mean (HM) , Fischer and Maurer in 1978 have worked on stress susceptibility index (SSI), Bet al.,ugh in 1984 have studied yield stability index (YSI), Gavuzzi and co-workers in 1997 have worked on yield index (YI), that recognize sensitive and endurance gene sequence accordance on the productions in non-tension and stress surroundings.

The most suitable choice indicators should identify gene sequence that has consistent perfection in non-tension and tension surrounding. Fernández in 1992 stated that mungbean (*Vigna radiata* L) gene sequence choice according to STI and GMP indicators lead to a gene sequence that has high endurance and product. Clarke and co-workers in 1992 utilized the SSI indicators for identifying among the wheat (*Triticum aestivum* L) gene sequence. In accordance with Sio-Se Mardeh and co-workers in 2006, STI, GMP, and MP were the most suitable indicators under average tension in wheat. The current research's purposes were an assessment of some dehydration endurance indicators also for identifying dehydration endurance gene sequence into the Durum wheat gene sequence.

MATERIAL AND METHODS

The material of herb and empirical arrangement 40 durum wheat reproduction lines (Triticum turgidum var. durum Desf.), were selected for the research in accordance with the important variations in product efficiency under the situation that are watered and non-watered. Investigations have been carried out in the empirical area of Islamic Azad University in Ardabil province, Iran in 2015 till 2018 (3 years of cropping). The empirical design was an accidental whole block plan by 3 irritation. Planting was accomplished by an empirical drill in 1.5 meters × 4 meters layout, including 5 lines 20 cm separate in four hundred grains m2 for every area. Manure was used to 41 kg ha^{-1} N and 46 kg ha^{-1} P_2O_5 and sowing was in accordance with the local soil examination recommendations before planting.

Watering was conducted in the non-tensioned area in the flowering step. For recognizing the physical and chemical features of soil examinations, samples of the soil before ground preparing processes have been conducted. Following the soil laboratory investigation and water laboratory investigation in the Islamic Azad University of Ardebil, samples of 0 till 30 and 30 till 60 cm deepness have been chosen; the outcomes in Table 2 are demonstrated (this examination was conducted solely for soil unity and for avoiding mistakes. in 60 cm wheat root entrance isn't needed for reviewing), and also the outcomes of Rain for 2015 till 2018 years are shown in fig 1 (WWO, 2018).

Dehydration endurance indicators were computed by utilizing the relations that are demonstrated as follows:

Tension severity was (SI=0.2).

Dehydration indicators: Dehydration endurance or sensitivity indicators have been computed for every

Genotype by utilizing the following relations:

1. (STI) = $[Ypi \times Ysi]/(Yp)^2$ (Fernandez, 1992)
2. (GMP) = $\sqrt{(Ypi \times Ysi)}$ (Fernandez, 1992)
3. (TOL) = $Ypi - Ysi$ (Hossain et al., 1990)
4. (MP) = $(Ypi + Ysi)/2$ (Rosielle and Hamblin, 1981).
5. (SSI) = $[1 - (Ysi - Ypi)]/SI$ (Fischer and Maurer, 1978);
6. (YSI) = Ysi/Ypi (Bet al.,ugh, 1984)
7. (YI) = Ysi/Ys (Gavuzzi et al., 1997; Lin et al., 1986)

Which, the cultivar in tension situation product is demonstrated by Ysi, and the cultivar in normal situation product is demonstrated by Ypi, the intensity of stress is demonstrated by (SI), that SI= $1Ys/Yp$; the whole yield average in stress situation is demonstrated by Ys, the whole yield average in the standard situation is shown by Yp. Through the water deficit indicators, more amount of SSI and TOL present almost extra susceptible for tension, therefore a minimum amount of SSI and TOL are desirable. The choice in accordance with the 2 principles chooses gene sequence by the potentiality of the lower product supporting non-tension situations and large product supporting tension situation. So, Fernandez in 1992 has demonstrated that the choice in accordance with GMP and STI would lead to gene sequences by more water deficit and the potentiality of the product would be chosen.

Statistical analysis: Variance Analysis, average comparing, the relationship among various methods and gene sequence group analysis in accordance with the distance of Euclidean was calculated using SPSS-25 and MSTAT-C softwares (SPSS, 2018). The Principal component analysis (PCA) was utilized for classifying the screen procedures also the gene sequence. According to the PCA, the display of bi-plot was as well as utilized for identifying endurance and large producing gene sequence utilizing Minitab16 software.

RESULTS AND DISCUSSION

Important variations between the gene sequences from the product aspect under non-tension and stress situations have existed (Table 3). Also, important variations between gene sequences have been seen for whole dehydration endurance indicators in the 0.01 possibility stage (Table 3). According to the attained outcomes, there exists a large genetic difference between gene sequences that can be an effective source for the dehydration endurance germplasm choice. The empirical variation coefficient (CV) altered from 3.48 to 23.18. Nonetheless, for a large number of features, the amounts were smaller than six percent (Table 2). Endurance indicators were computed in accordance with the GY of the gene sequences (Table 4). Large product amount in tension and non-tension surroundings was presented by gene sequences 'NO. 40 (4411.22 Kg ha^{-1}) and 'NO.32' (4256.34 Kg ha-1) orderly (Table 4). The highest STI amount (1.07), MP (3642.11) and GMP (3590.85) indicators were with gene sequence 'NO.35'. The max YI amount (1.24) was from gene sequences 'NO.39' and 'NO.21' (Table 4). In the following investigation, regression of generic linear model of GY under dehydration tension on YSI showed a positive association among these criteria by a similar determining ratio (R2= 0.83).

Golabadi and co-workers in 2006 and Talebi and co-workers in 2009 have demonstrated that choice according to a composition of indicators could produce so effective criteria to improve the wheat dehydration endurance however, association ratios are effective for finding the general linear degree correlation among every 2 properties. Therefore, a more desirable method in comparison with an association analysis like a bi-plot is required for identifying better gene sequences for non-tensioned and tensioned surroundings. for identifying the better indicators of choice for dehydration endurance gene sequences, the association ratio among these indicators as well as yield in the normal irrigation situation (YP) also yield in the stress situation (YS) was defined (Table 5).

Table 1. Durum wheat genotypes and regions.

NO.	Genotype	NO.	Genotype
1	Hordeiforme	21	Africanum
2	Africanum	22	Leucurum
3	(Omrabi15)	23	Hordeiforme
4	Leucurum	24	leucumelan
5	Melanopus	25	Niloticum
6	Hordeiforme	26	Africanum
7	Leucurum	27	Boeuffi
8	Leucurum	28	Leucumelan
9	Melanopus	29	Apulicum
10	Leucurum	30	Erythromelan
11	Reichenbachi	31	Barakatly-95
12	Saiymareh	32	Sharq
13	Hordeiforme	33	Hordeiforme (Ahar)
14	Apulicum	34	Apulicum
15	Boeuffi	35	Apulicum
16	Leucumelan	36	Africanum
17	Melanopus	37	Melanopus
18	Albiprovinciale	38	Boeuffi
19	Murceinse	39	Melanopus
20	Leucurum	40	Apulicum

Matrix of association ratios (Table 5) showed thatGMP, MP, STI, YI and TOL indicators can efficiently be utilized to screen of dehydration endurance gene sequences. SSI and TOL under rainy situation were negatively and extremely remarkably (P<0.05) associated with Ys (Table 5). Richards in 2002, Van Ginkel and co-workers in 1998, Rajaram and Van Ginkel in 2001, Betran and co-workers in 2003 determined 2 principal classes of the theory herb growers that aim their germplasm for dehydration-prone regions. The 1-st theories declare which large input receptivity and genetically large producing potentiality,

composed by tension-adaptive features would ameliorate efficiency in the dehydration-influenced surrounding.

PCA (principal component analysis) showed that the 1-st PCA revealed 59.3 percent of the whole information variety and possessed positive association with the function under non-tension and tension surroundings (Table 6). Therefore, the 1-st dimension presents the product potentiality and dehydration endurance. We can state that this element could divide the gene sequences by more product under non-tension and tension situations. The 2-nd PCA revealed 39.9 percent of whole information variety (Table 6). The 1-st 2 PCAs considered for approximately 99.2 percent of the whole variety. PCA showed the indicators can distinguish the wheat gene sequences. Bi-plot representation described gene sequences NO‘ 18, 22, 17, 23, 39, 6, 25, 19, 16, 30, 33, 10, 35, 32, 40, 1, 36 and 21’ placed near to significant dehydration endurance indicators which verify the gene sequences being dehydration endurance.

Table 2. Soil analysis outcomes

Soil type	Soil texture			Absorbent Potassium ppm))	Absorbent Phosphorus (ppm)	Total nitrogen (%)	Organic carbon (%)	Neutral-reacting material (%)	(PH)	Electrical conductivity (ds/m)	Saturation	Depth (cm)
	Sand	Silt	Clay									
Clay	40	36	24	290	2	0.056	0.47	7	8.2	2.4	45	.0-60
Clay loam	31	41	28	460	4.8	0.103	0.97	4.8	7.8	2.66	48	0-30

Table 3. The average of seed production of genotypes gene sequence under both conditions

Source of variation	Mean Square									
	df[1]	YP[2]	YS[3]	SSI[4]	TOL[5]	MP[6]	GMP[7]	STI[8]	YI[9]	YSI[10]
Genotypes	39	6427.2**	4462.2**	0.9**	5588.1**	4047.5**	4115.6**	0.103**	0.067**	0.04**
Year	2	8247.0**	1314.1**	1.2*	1097.0**	2291.3**	1810.8**	0.46**	0.003**	0.4**
Error	78	1.42	1.52	0.06	4.17	2.01	2.02	0.002	0.0004	0.001
CV (%)11	-	3.9	4.5	23.1	6.9	3.4	5.5	5.3	5.1	4.5

[1]df: degrees of freedom. [2]YP: Yield of a proposed gene sequence in optimum (potential) situations. [3]YS: Yield of a proposed gene sequence in stress situations. [4]SSI: stress susceptibility index. [5]TOL: tolerance index. [6]MP: mean productivity. [7]GMP: geometric mean productivity. [8]STI: stress tolerance index. [9]YI: yield index. 10 YSI: yield stability index. 11 CV: coefficient of variation. **: significant at 0.05 and 0.01 probability level, respectively.

Gene sequence NO‘ 12, 3, 7, 8, 37, 1, 14, 15, 4, 5, 27, 11, 20, 28, 13, 24, 29 and 26 was approaching to SSI and has large YP (grain product in non-tension situation) amount. Hence, this gene sequence had special versatility to the non-tension surrounding. Gene sequence No. 34, belong to minimum product and maximum dehydration sensitivity area in the bi-plot region. There existed genetic variation amongst gene sequences in accordance with their dehydration endurance. Utilizing significant endurance indicators including MP, GMP, STI, HM, YI and TOL gene sequences UPGMA assortment was conducted and 3 groups were created which matched the bi-plot analysis outcomes. As well as the cluster Dendrogram outcomes verified the main element analysis outcomes.

The product amounts of CV in the non-tension situation were 3.94 and the product in tension situation were 4.51. Regarding computed indicators, the amounts changed between 3.48 and 23.18 (Table 2). generally, CV amount more than twenty percent is supposed to be large; nevertheless, could be probable for ignoring from about large CV amounts while F experiment is important and the aforementioned case is observed in some distributed searches (Takemoto et al. in 1988; Xu et al. in 2000; Aliyu and Awopetu in 2005; Zarei et al. in 2007; Okwuagwu et al. in 2008; Kandiç et al. in 2009; Sabu et al. in 2009). Nevertheless, Aliyu and Awopetu in 2005 have shown that the gene sequences impact was so noticeable on considered cases under 2 regimes of watering. Some test has accomplished by Okwuagwu and co-workers in 2008 that has demonstrated that the opposite CV amounts stated in several investigations as our one may be because of physio-genetic features and adaptability degree of the material of herb, small amount of particular each gene sequence in plant, small amount of iteration each gene sequence and/or unsteady surroundings.

Variety owing to gene sequences was important for whole features in 2 situations (rainy and badly watered). This finding proposed that the measure of variations in

gene sequences was adequate for providing some range to select gene sequences for improving dehydration endurance. The average comparison illustrated that G40 possessed the maximum GY amount. Product and product-associated features under water deficit conditions were not dependent on the product and product-associated features under non-tension situations; however, this wasn't the item in min intense tension situations. Since STI, GMP and MP can be able to distinguish cultivars generating a great product in two situations.

Table 4. mean product Durum wheat gene sequence under optimum and tension situations, and computed various dehydration endurance indicators[1].

NO	YP	YS	STI	MP	TOL	GMP	SSI	YSI	YI
1	3768.83	3063.08	0.95	3415.96	705.75	3393.94	0.75	0.82	1.19
2	2942.64	2494.36	0.61	2718.51	448.28	2706.25	0.6	0.85	0.97
3	3046.58	2339.81	0.58	2693.2	706.78	2663.03	0.9	0.78	0.91
4	3302.26	2487.56	0.67	2894.92	814.7	2859.45	0.98	0.76	0.97
5	3211.39	2409.16	0.63	2810.28	802.23	2775.37	0.99	0.76	0.94
6	3707.39	2857.8	0.87	3282.6	849.58	3248.95	0.92	0.78	1.11
7	3052.22	2291.72	0.58	2671.97	760.5	2639.29	1	0.76	0.89
8	3155.38	2436.52	0.63	2795.96	718.86	2765.7	0.88	0.78	0.95
9	2856.83	1668.52	0.39	2262.68	1188.3	2176.71	1.74	0.6	0.65
10	4174.59	3097.8	1.07	3636.2	1076.78	3586.61	1.07	0.75	1.2
11	3449.46	2364.17	0.67	2906.82	1085.29	2847.13	1.28	0.7	0.92
12	3158.78	2356.52	0.62	2757.66	802.26	2723.99	1.04	0.75	0.92
13	3214.58	2142.92	0.56	2678.76	1071.66	2618.26	1.38	0.68	0.83
14	3172.99	2308.52	0.6	2740.76	864.47	2699.68	1.09	0.74	0.9
15	3170.94	2308.04	0.61	2739.5	862.9	2701.17	1.13	0.73	0.9
16	3496.98	2809.8	0.81	3153.4	687.18	3128.07	0.76	0.81	1.09
17	3905.79	3104.2	1	3505	801.58	3476.16	0.81	0.8	1.21
18	3812.19	2643.72	0.83	3227.96	1168.47	3169.12	1.28	0.7	1.03
19	3513.46	2847.08	0.82	3180.27	666.38	3156.22	0.73	0.82	1.11
20	3539.06	2265.8	0.66	2902.43	1273.26	2826.1	1.52	0.65	0.88
21	3864.19	3193.8	1.02	3529	670.39	3507	0.67	0.83	1.24
22	3751.54	3172.52	0.98	3462.04	579.02	3443.6	0.57	0.85	1.23
23	4152.51	2593.96	0.89	3373.24	1558.54	3277.21	1.61	0.63	1.01
24	3240.98	2249.16	0.6	2745.07	991.82	2693.45	1.25	0.7	0.87
25	3796.19	2909.32	0.91	3352.76	886.87	3317.42	0.94	0.77	1.13
26	3422.1	2248.04	0.63	2835.08	1174.06	2767.7	1.44	0.67	0.87
27	3237.79	2402.92	0.64	2820.36	834.86	2782.57	1.03	0.75	0.93
28	3924.19	2233.8	0.72	3079	1690.39	2955.95	1.86	0.57	0.87
29	3156.23	2248.84	0.58	2702.54	907.38	2658.91	1.18	0.72	0.87
30	3351.06	2716.36	0.75	3033.71	634.7	3010.22	0.71	0.82	1.06
31	4032.5	1796.52	0.6	2914.52	2235.98	2687.58	2.43	0.45	0.7
32	4256.34	2846.6	1.01	3551.48	1409.73	3478.18	1.43	0.67	1.11
33	3541.46	2864.04	0.83	3202.75	677.42	3178.3	0.73	0.82	1.11
34	2308.19	2901.32	0.54	2604.76	-593.14	2575.28	-1.52	1.3	1.13
35	4246.1	3038.12	1.07	3642.11	1207.97	3590.85	1.19	0.72	1.18
36	3988.19	2751.56	0.9	3369.88	1236.62	3307.39	1.3	0.7	1.07
37	3101.14	2392.52	0.61	2746.84	708.62	2716.72	0.88	0.78	0.93
38	2924.19	1993.16	0.48	2458.68	931.02	2407.16	1.29	0.69	0.78
39	3912.63	3188.84	1.03	3550.74	723.78	3528.12	0.75	0.82	1.24
40	4411.22	2818.76	1.03	3615	1592.46	3521.65	1.55	0.64	1.1

1 indicators: see Table 3.

While the tension has been intense, TOL, YSI, and SSI have been seemed to be so effective indicators separating endurance cultivars, however, any of indicates cannot definitely recognize cultivars by the large product under non-tension and tension situations (cluster A cultivars). Blum in 1996 has concluded which the choice indicators

productiveness relies on the intense of the tension under the opinion which solely supporting average tension situations, potentiality product considerably affects product supporting tension.

The growers that support choice in desirable surroundings obey this theory. Yielders, hence, would rather cultivars which generate large productions while water isn't such restricting, however, Nasir Ud-Din and co-workers in 1992 have found that suffering the smallest damage throughout dehydration seasons. The 2-nd idea is which improvement in product and adoption in dehydration-influenced surroundings could be obtained solely with choice under the prevalent situations discovered in purpose surroundings (Ceccarelli in1987, Ceccarelli and Grando in 1991and Rathjen in 1994). Falconer in1952 has given the theoretic system for this subject that Falconer has written, "Product in the min and max producing surroundings could be regarded as divide features that aren't certainly enlarged with same allele's collection".

Table 5. The association among various dehydration endurance indicator 1 and an average production of Durum wheat gene sequence under optimum and tension situations

	YS	YP	GMP	MP	TOL	SSI	STI	YI	YSI
YS	1								
YP	0.495**	1							
GMP	0.88**	0.846**	1						
MP	0.837**	0.890**	0.995**	1					
TOL	-0.363*	0.63**	0.121	0.207	1				
SSI	-0.514*	0.455**	-0.054	0.017	0.947**	1			
STI	0.873**	0.849**	0.998**	0.994**	0.130	-0.043	1		
YI	1.00**	0.495**	0.88**	0.837**	-0.362**	-0.515**	0.873**	1	
YSI	0.252**	-0.444**	0.067	-0.004	-0.945**	-1.00**	0.056	0.525**	1

1 indicator: see Table 3. ** And *: important at 0.01 and 0.05 possibility stages.

Table 6. Eigen amount and main element analysis vectors for potential yield (YP), stress yield (YS) and dehydration endurance indicator[1].

Principal component	1	2
Percentage of variance	59.3	39.9
Cumulative percentage	59.3	99.2
YS	0.41	-0.16
YP	0.32	0.35
GMP	0.42	0.09
MP	0.41	0.13
TOL	-0.02	0.52
SSI	-0.10	0.50
STI	0.42	0.10
YI	0.41	-0.16
YSI	0.10	-0.50

1 Indices: see Table 2.

In general, dehydration tension diminished remarkably the product of several gene sequences and some can be shown endurance for dehydration that in this material, proposed the genetical variations for dehydration endurance. Hence, according to the restricted model and surroundings, examination and determination under tension and non-tension situation solely can't be so efficient for enhancing production under dehydration tension. The positive and important association of Yp and MP, STI and GMP demonstrated that these indicators were so efficient in recognizing large producing cultivars under various precipitation situations. The estimated improve outcomes from the devious choice in precipitation tension surrounding could gain production in precipitation tension surrounding more desirable in comparison with a choice from the non-precipitation tension surrounding. So, wheat growers must consider the surrounding tension intensity while selecting an indicator. Ultimately, we can understand from these studies that gene sequence No. 10 and 35 in accordance with STI, Mp and GMP indicators were endurance gene sequence and these gene sequences are helpful for dehydration endurance choice.

in the time of gathering, for preventing boundary impact, fifty cm of every line from two sides were deleted for gathering and calculating Plot production.

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Phytoadaptogen Effect Based on *Glycyrrhiza glabra* on Adolescent Body Adaptation Capabilities Living Under Conditions of Environment Chemical Pollution

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ABSTRACT

According to various sources, 15–20% of the world territories, are the zones of ecological disaster, about 50% of the population live in environmentally unfriendly regions. Licorice medicinal plant with the scientific name of *Glycyrrhize globra* is one of the medicinal plants used in cosmetics, food, health and pharmaceutical industries. The main aim of the study is to present the data on the correction of deviations concerning the adaptation system activity of 13-14-year-old adolescent bodies, exposed to chemical pollution of the environment by means of a phytoadaptogen based on licorice. According to the obtained data, they established the reduction of chemical, environmental pollution factor negative effect on the adolescent's body: normalization of rhythm, blood pressure, heart rate, maximum oxygen consumption, and the adaptive potential of the circulatory system.The results of the study demonstrate that the use of *Glycyrrhiza glabra* root extract as an adaptogen can help the normalization of blood pressure, heart rate, and variation range among 14-year old adolescents living in conditions of environmental chemical pollution. Besides, it can be concluded that *Glycyrrhiza glabra* root extract is an positive natural adaptogen since it decreases the detrimental impact of chemical pollution factors on the adolescent body, and consequently results in relative normalization of blood pressure.

KEY WORDS: ADOLESCENTS, ADAPTATION OPPORTUNITIES, CHEMICAL POLLUTION OF THE ENVIRONMENT, PHYTOADAPTOGENS, PHOTO CORRECTION, LICORICE.

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INTRODUCTION

In recent decades, a large amount of scientific information has appeared about the influence of an unfriendly environment on the physical development and functional state of a child's body (Agadzhanyan et al., 1993; Baevsky et al., 1987; Veltishchev and Fokeeva, 1996; Guminsky et al., 1990; Resenkova, 2003; Tolstikov et al., 1991; Ghorbanlou et al., 2020). The most acute issue is the

need for effective measures aimed at body resistance increase in adverse environmental conditions. For many centuries, various medicinal substances have been used by medicine for the treatment and rehabilitation of humans. In recent years, some low-toxic biologically active herbal remedies are purposefully used in sports practice to accelerate recovery, actively replenish spent plastic and energy resources, and selectively control the most important functional systems of the body during heavy physical exercise (Brehman, 1969; Yermolova and Zimova, 2001; Kerimov and Kasumov, 1998; Resenkova, 2003; Sidorova et al., 2020).

Table 1. Child physical performance assessment in terms of MOC/kg (A.A. Guminsky et al., 1990)

Age, years	MOC, ml/min•kg		Evaluation
	Girls	Boys	
13	41,0 43,0 45,0	37,5 39,5 41,5	low satisfactory high
14	43,6 45,5 47,5	35,5 37,5 39,5	low satisfactory high

Adaptogens are important among them. Adaptogens are the substances that have a general tonic effect on the body and increase its resistance during heavy physical exertion, under hypoxic conditions, and during drastic bioclimatic changes (Butova, 1999). Plant adaptogens can stimulate the nervous system and metabolic processes in the body moderately, which has a beneficial effect on the adaptation to physical exertion. Currently, herbal preparations based on *Glycyrrhiza glabra* root are in the field of study by modern scientists (Brehman, 1969; Yermolova and Zimova, 2001; Resenkova, 2003; Streltsov, 2002). A number of authors indicate the presence of anti-toxic (Cekic et al., 2012; Milashechkina, 2005) and immunomodulating (Ishida and Sympos, 1983; Halberg, 1969; Singamaneni et al., 2020) properties of licorice root.

Therefore, the goal of our study is to evaluate the effect of the drug on the basis of *Glycyrrhiza glabra* on the leading adaptation systems of the adolescent body living in conditions of chemical, environmental pollution. *Belyaev* (1969), Rezenkova (2002), Milashechkina (2003) studied the effect of *Glycyrrhiza glabra* extract on the processes of the organism adaptation. The authors found that the *Glycyrrhiza glabra* root has the obvious adaptogenic effect, which is reflected in the harmonization of the hormonal balance and, thus, the stimulation of adaptive responses to environmental factors.

Table 2. Evaluation of organism functional capabilities according to the values of the circulatory system adaptive potential

AP (in the cond. score)	Assessment of adaptation degree	Functionality level	Recommendations and activities
<1.60	Satisfactory	Optimal	Therapeutic
1.60-2.09	Incomplete or partial	Sufficient	Therapeutic
2.10-2.59	Unstable	There is a risk of decline	Improving and preventive
2.60-3.09	Stress adaptation mechanisms	Reduction	Preventive and curative
>3.10	Unsatisfactory, overstrain of adaptation mechanisms	A sharp decline	Medicinal

The confirmation is the increase of physical performance, the resistance to hypoxia and better results in the development of physical qualities - general physical endurance. Also, the *Glycyrrhiza glabra* extract implements its adaptogenic properties by optimizing the functional state of the central nervous system, helping to balance excitatory and inhibitory processes and to improve its quantitative and qualitative characteristics (Sadek et al., 2020) These data gave rise to the use of *Glycyrrhiza glabra* root extract as a stress-limiting agent among a group of 14-year-old teenagers, in which most of the indicators revealed the most significant negative changes.

MATERIAL AND METHODS

The influence of chemical, environmental factors on the body of adolescents was studied in natural experiment conditions. The participants of the study were 13-14-year-old adolescents, who were divided into the following groups: 1) control group (n=82; 42 boys and 40 girls) which are living in the area without anthropogenic pressure; 2) test group (n =102; 61 boys and 41 girls) which are living in the conditions of environmental chemical pollution; 3) correctional group (n=37), with the use of licorice root extract - it consisted of 17 boys and 20 girls living in the conditions of environmental chemical pollution.

A phytoadaptogen, based on *Glycyrrhiza glabra* extract, was given in the morning from 7.30 to 8.30 at the dose of 0.05 mg/kilogram of body weight (Resenkova, 2003). Also, to determine the adaptive capacity of the adolescent body, we used the indicators characterizing the state of regulatory mechanisms. The state of the respiratory system was determined by relative and absolute lung capacity

(LC), chest excursion and maximal oxygen consumption (MOC) test, the state of the circulatory system and its regulatory mechanisms by cardiointervalography indices: HRV (heart rate variability), arterial pressure (AP), the adaptive capacity of the circulatory system and an individual minute duration.

The stress test recognized by the World Health Organization as an objective and informative indicator of the functional status of the cardiorespiratory system and the functionality of a person - the maximum oxygen consumption (MOC). According to the researchers, the indirect method for the determination of MOC was proposed by A.A. Guminsky (Dzhandarova et al., 2014). The evaluation of the test results was carried out according to the data presented in Table 1. The adaptive potential of the circulatory system was determined by R.M. Baevsky's method (Belyaev, 2002), adapted for the use on the child organism by P.A. Fileshy (Resenkova, 2003), the assessment was made in accordance with the data given in Table 2.

Table 3. The state of the cardiorespiratory system among 14-year-old adolescents living in a chemically contaminated area after photo correction

Indicators	Control	Test	P1	Correctional group	P2	P3
		Girls				
VCL, l	2,38±0,05	2,27±0,08	>0,05	2,32±0,12	>0,05	>0,05
SP, mm.m.col.	99,29±1,70	109,70±1,70	<0,01	109,29±4,90	<0,05	>0,05
DP, mm.m.col.	59,29±0,29	63,77±1,52	<0,05	67,57±3,16	<0,01	<0,01
HR,b/min	81,77±3,01	92,2±3,63	<0,05	85,81±4,36	>0,05	>0,05
		Boys				
VCL, l	3,34±0,05	2,29±0,15	<0,001	2,32±0,16	<0,001	<0,001
SP, mm.m.col.	99,17±1,52	121,26±2,59	<0,001	107,33±1,75	<0,001	<0,01
DP, mm.m.col.	57,92±0,52	76,6±1,34	<0,001	66,33±5,50	>0,05	>0,05
HR,b/min	81,45±3,52	86,78±2,00	>0,05	88,00±1,39	>0,05	>0,05

Note: P1 - the reliability of differences in average values between the experimental and control groups; P2 - the reliability of differences in average values between the experimental and correction groups; P3 - the reliability of differences in average values between the control and correction groups.

Table 4. Heart rate variability among 14-year-old adolescents living in a chemically contaminated area, after photo correction

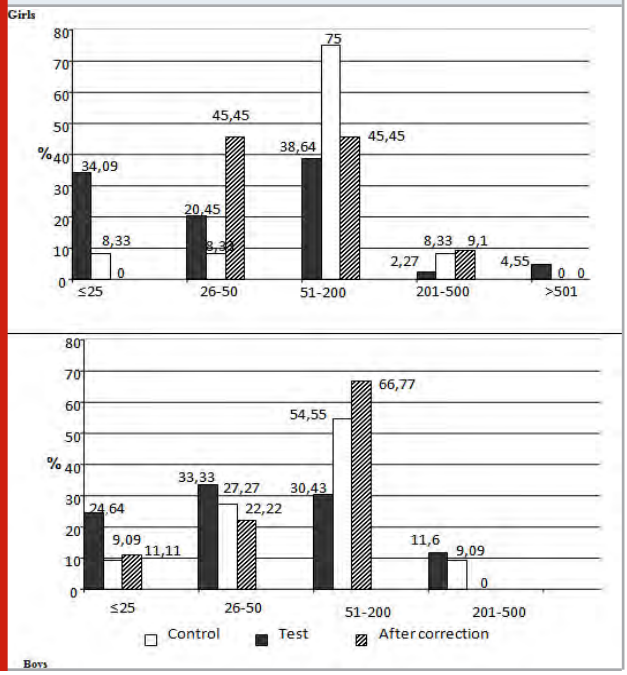
Indicators	Control group	Test group	P1	Correction group	P2	P3
		Girls				
M, ms	743±27,90	673,45±17,34	<0,05	715,45±46,79	>0,05	>0,05
MSD, ms	58,33±5,89	76,82±4,66	<0,05	66,45±9,50	>0,05	>0,05
ΔX, ms	331,33±30,23	649,70±61,54	<0,001	372,00±47,77	<0,001	>0,05
Mo, ms	716,67±33,22	647,73±18,84	>0,05	704,55±58,74	>0,05	>0,05
Amo, %	36,83±3,40	36,25±2,19	>0,05	34,00±2,84	>0,05	>0,05
		Boys				
M, ms	747±33,19	707,67±20,46	>0,05	683,56±10,64	>0,05	>0,05
MSD, ms	64,82±12,56	68,99±3,40	>0,05	66,44±10,53	>0,05	>0,05
ΔX, ms	362,91±51,36	563,90±31,65	<0,01	421,00±64,56	<0,01	>0,05
Mo, ms	731,81±42,80	673,91±19,05	>0,05	683,33±17,54	>0,05	>0,05
AMo, %	35,36±4,19	36,10±1,62	>0,05	35,44±2,92	>0,05	>0,05

Note: P1 is the reliability of differences in average values between the experimental and control groups; P2 is the reliability of differences in average values between the experimental and correction groups; P3 is the reliability of differences in average values between the control and correction groups.

The vital capacity of the lungs (VCL), expressed in litres, was measured using a spirograph. The subject drew the maximum inhale and then gradually exhaled the air through the mouthpiece into the spirograph. Cardio intervals were recorded and analyzed using the Varicard automated computer instrument. Statistical characteristics

of the dynamic range of cardio intervals included: expectation (M), heart rate (HR) and standard deviation (σ), expectation (M). The numerical characteristics of variational pulsograms along with indicators of statistical estimates were mode (Mo), variational span (Δx) and mode amplitude (AMo). Individual minute (IM) was determined by F. Halberg's method. According to the author method, the value of IM is a fairly informative test. The magnitude of myocardial infarction is a relatively stable indicator among healthy people. Mathematical-statistical processing of the survey results was carried out using Microsoft Excel software. The level of different significance for the studied parameters was determined using Student's criterion. The results were considered statistically significant at $p \leq 0.05$.

Figure 1: IN in st. units among 14-year-old girls and boys, living in a chemically polluted area, after photo correction with *Glycyrrhiza glabra* root extract



RESULTS AND DISCUSSION

According to the data we obtained (Table 3), when you use a phytoadaptogen based on *Glycyrrhiza glabra* VCL tends to increase among boys and girls, and SP has decreased significantly among boys (P<0,05), girls show the tendency of its decrease. This can be explained by the fact that the boys had more pronounced changes in blood pressure indicators as compared with girls, so they were also more sensitive to the effects of phytoadaptogen. The intake of *Glycyrrhiza glabra* root extract contributed to the normalization of the cardiovascular system functional capabilities (Table 4). The indicators of the expectation and heart rate variation decreased significantly among girls and boys, approaching the control group, as compared with the original (P>0.05).

Analyzing the indicators of IN before and after *Glycyrrhiza glabra* extract application (Fig. 1), they revealed the increase of adolescent number with a state of vegetative balance, both among boys (66.77%) and among girls (45.45%), which indicates the "smoothing" of chemical pollution negative impact after the use of phytoadaptogen based on *Glycyrrhiza glabra* root. Moreover, 20.3% of adolescents from the correctional group, had the value of IN even lower than in the control group, which, together with the change of expectation and variation scope indicators, indicates a high sensitivity of the vegetative nervous system to this phytoadaptogen. They revealed the positive effect of *Glycyrrhiza glabra* extract on the level of adolescent body adaptive abilities (Table 5). Thus, the relative magnitude of MOC/kg improved by 1.26 times among girls, and by 1.32 times among boys and approached that in the control group. The same can be said about the indicators of the circulatory system adaptive potential and the duration of an individual minute (Table 5). After correction, the average values of AP decreased and varied within the limits of a sufficient level of organism functional capacity of the organism (AP=1.61-2.09) both among girls and boys, averaging 1.93 ± 0.26. There has been an increase in individual minute duration.

Table 5. Adaptive abilities of 14-year old schoolchild body, living in a chemically contaminated area, after photo correction

Indicators	Control group	Test group	P1	Correction group	P2	P3
		Girls				
MOC/kg, ml./min./kg.	45,84±0,40	36,17±0,62	<0,001	45,45±1,29	<0,001	>0,05
AP, st.un.	1,75±0,03	2,27±0,08	<0,001	2,05±0,09	>0,05	<0,05
IM, s	45,56±0,02	56,91±0,99	<0,001	49,55±3,52	<0,05	>0,05
		Boys				
MOC/kg, ml./min./kg.	47,79±0,42	45,17±0,51	<0,001	46,83±1,09	>0,05	>0,1
AP, st.un.	1,76 ±0,03	2,22±0,05	<0,001	1,99±0,07	<0,001	<0,05
IM, s	54,68±0,11	45,90±0,53	<0,001	49,50±2,62	<0,05	>0,05

Note: P1 is the reliability of differences in average values between the experimental and control groups; P2 is the reliability of differences in average values between the experimental and correction groups; P3 is the reliability of differences in average values between the control and correction groups.

In this study, the data on the correction of deviations concerning the adaptation system activity of 13-14-year-old adolescent bodies, exposed to chemical pollution of the environment by means of a phytoadaptogen based on *Glycyrrhiza glabra* was investigated. The outcomes revealed that the application of *Glycyrrhiza glabra* root extract as an adaptogen contributed to the normalization of blood pressure, heart rate, and variation range among 14-year old adolescents living in conditions of environmental chemical pollution, (Sadek et al., 2020). Moreover, more pronounced changes towards the normalization of the functional state of the cardiorespiratory system were found among boys, which is probably explained by their more pronounced changes before correction. Among the adolescents of the correctional group, the level of adaptive capacity has increased, as was indicated by AP and MOC indicators. The indicators of IM approached those of the control group, which indicates the stabilization of rhythmostasis (Sidorova et al., 2020).

CONCLUSION

The study found that *Glycyrrhiza glabra* root extract is an effective natural adaptogen because it reduces the negative effect of chemical pollution factors on the adolescent body: it leads to a relative normalization of blood pressure, heart rate indicators, individual minute and maximum oxygen consumption. This provides a scientific basis for the development and the use of herbal remedies based on licorice as adaptogens, in order to level the negative effects of environmental chemical pollution.

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Utility of Foldscope in Promoting Scientific Temper by Igniting the Mind: A Study Among Rural and Urban Senior Secondary School Learners

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ABSTRACT

It is impossible to identify various microorganisms present in our local environment at the spot without sampling in laboratory. The Foldscope is an origami portable, optical and affordable microscope which is more comfortable than the conventional microscope for field studies. Moreover, it can be used as a high-end frugal technology for teaching science. The exposure of students to Foldscope in biology courses could channel student's interest towards further experimental activity and original research. This study aims to explore the usability of the foldscope in science classroom and additionally in their projects and field survey as per their syllabus. For this study 600 students (300 from rural and 300 from urban) were selected randomly as sample from 30 secondary schools (15 from rural and 15 from urban) in Lakhimpur district of Assam. A comparison on utilizing scientific temper in the experiments was made between the schools of urban and rural areas. Workshops cum handling sessions were successfully conducted among both the teachers and students. Water samples including, bacteria, algae and insects body parts were observed by the students. Statistical analysis of the data in respect of different dimensions of scientific temper such as curiosity, open-mindness, objectivity, rationality, aversion to superstition was also performed for both rural and urban secondary school students and the result showed that the students of rural schools were more curious, logistic and interactive regarding the instrument. This study was, in short, quite successful in approving our predetermined results. The concept of 'aversion to superstition' was fulfilling enough to apply.

KEY WORDS: FOLDSCOPE, SCIENTIFIC TEMPER, SECONDARY STUDENTS.

INTRODUCTION

Education and Research is nowadays a term up solution that sound to resolve any impact to the well being. Adequate research and tools are now a synchronized matter of concern in this scientific world. To bring up the socio-economic development and awareness education is only the key to drive a society. Development of technology, pedagogy, and content knowledge with respect to science teachers shows a manageable contact among the teachers and students (Guzey et al., 2009). In India the government schools have been affected in their education process due to lack of well-equipped infrastructure (Lahon, 2015). To cope up with a vast of knowledge practical rather than theory is easier to devour but is complexed in our system (India) due to lack of skilled teachers, well equipped infrastructure and technologies (Kro, 2017). A recent investigation report that a new way of teaching learning procedure is equipped by online interactions,

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group conference and projects that develops a framed knowledge in better understanding among the students (Tal et al., 2018).

Mobile has also been a tremendous device in learning process that seems to provide awareness, conservation of samples and even statistical analysis (Kissi et al., 2018). Microscopic world proves to be an elevated level of interest in science. The best effort of understanding biological science is the use of lens and resolution that provides an image beyond its imagination. Students and teachers are found to be more interactive and engaged in their experiment, by the use of microscope in classrooms. Visual information provides a quick action in student's brain, helping to memorize the facts and figures. Fast learning of biological science is more motivational by hands on training, collection of samples, preparations of slides, questioning and discussions (Mirko et al., 2018).

A study in various schools in Split-Dalmatia Country reported that 53% teachers used microscope for quality teaching of biological experiments while the rest have a reason of insufficient availability of microscope, lack of space and trained teachers (Mirko et al., 2018). Student positive reviews show a graceful advantage of using foldscope in government and private schools in understanding the biological experiments (Das et al., 2019). Morphological and characterization of fungal pathogens in tea leaves of Sikkim were also being observed. Different crucial stages of the malaria parasite *Plasmodium falciparum* was lucratively identified under the foldscope. Students mind seems to be full of curiosity (Wangdi et al., 2019; Shailaja et al., 2019).

Scientific temper promotes a handful understanding beyond in experiments of the subjects enhancing logic, interactions, observations and open mindness (Thakur et al., 2019). An interesting throw in Genetics to study the growth stages and mutants in Drosophila under foldscope proves a majestic success in both schools and colleges. This study mainly focused on the handling and use of foldscope in schools and colleges of Lakhimpur district, Assam. Workshop cum handling session was successfully conducted among both teachers and students. Students were allowed to carry the foldscope to the field work for observations of samples including water bacteria, algae, plant cells and different body parts of insects and images were stored in smart phones (Walling et al., 2020). Observation of bacteria in water samples including *E. coli, Acinetobacter* were also done by the college students as per their project work in the end semester.

MATERIAL AND METHODS

The Foldscope is an ultra-design origami microscope (Fig 1) signifying the conventional microscopes, can be carried to harsh field conditions which can be affordable by schools and colleges for biological experiments (Cybulski et al., 2014). This origami instrument is extensively used for the purpose of fieldwork in Sandy Hook New Jersey. Different water and sand samples were observed and images are been stored in smartphones (Bazler et al., 2016). It can be assembled from a punched sheet of cardstock, a spherical glass lens, a light emitting diode and a diffuser panel, along with a watch battery that powers the LED. The use of foldscope by students is bearable due to less cost, non breakable and easy to handle. The foldscope can be used by the student of any age to visualize the samples and is phone fitted (Buragohain et al., 2019).

The study area for research, Lakhimpur district (Fig 2) lies on the North East corner of Assam and at the North Bank of the mighty river Brahmaputra. The district lies between 27.597' Northern latitude and 94.737' Eastern longitude and covers an area of 2277 Sq km (Statistical Hand Book, 2017). For this study 600 students (300 from rural and 300 from urban) were selected randomly as sample from 30 secondary schools (15 from rural and 15 from urban) in the study area. The objective of this study had been formulated to explore the use of foldscope in the education institutions of the study area, to study the various dimensions of scientific temper of rural and urban secondary school students and their comparative study. For the preparation of sample, students were allowed to carry the foldscope in the fields survey for collection of samples and prepare the slides at instant. The visualization of the samples was observed and pictures were also stored in smart phones by the teachers (Buragohain et al., 2019).

Figure 1: The Foldscope

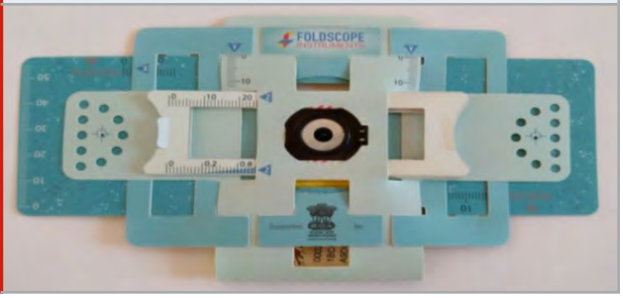
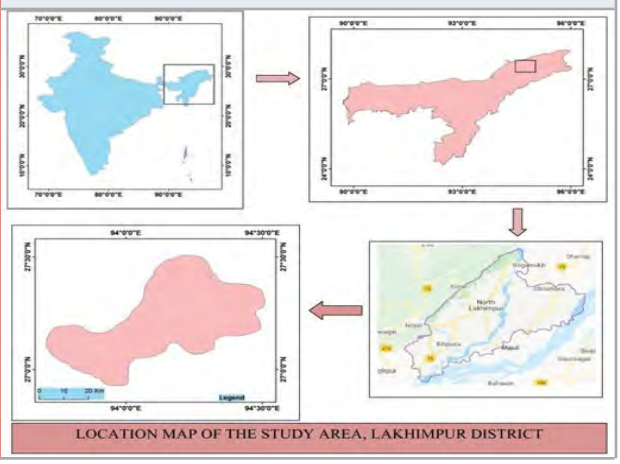


Figure 2: A cross sectional view of the study area



RESULTS AND DISCUSSION

Various organized workshops on hands-on-training and handling sessions regarding applications of foldscope were successfully conducted in different educational institutes among the students and the teachers (Fig 3a - 3e).

Figure 3a: Workshop on handling of foldscope in Asian Public School, North Lakhimpur

Figure 3b: Workshop on handling of foldscope in Genius Academy, North Lakhimpur

Figure 3c: Workshop on handling of foldscope in Laluk H.S. School, Laluk

Figure 3d: Workshop on handling of foldscope in Bodoti Jamuguri Janajati High School, Bihpuria

Figure 3e: Workshop on handling of foldscope in Lakhimpur Girls' College, North Lakhimpur

The slides for foldscopic images of isolated bacteria were prepared by simple staining for selected colonies. On the basis of cell wall composition to differentiate the bacteria whether it belongs to Gram positive or Gram negative, Gram's staining was done (Fig 4a) and the visualization of clinical isolates under foldscope was done (Fig 4b). Bacterial cultures were observed to find gram negative and gram positive bacteria by performing staining at various time intervals. The slide was placed in the paper based microscope interfaced with a mobile phone. After staining the cells were observed under foldscope and recorded the images for further analysis (Fig 4c).

Figure 4a: Microbial growth on nutrient agar plates

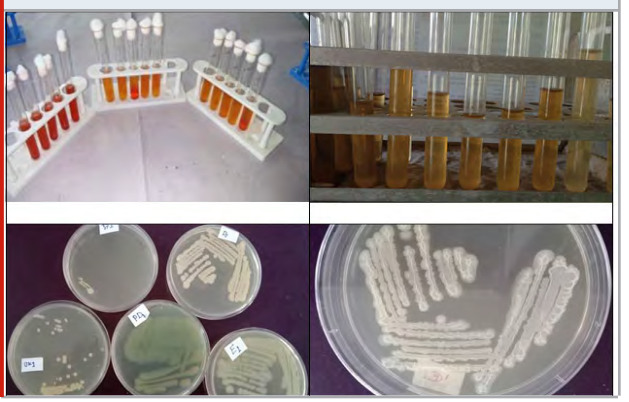


Figure 4b: Visualization of clinical isolates under foldscope

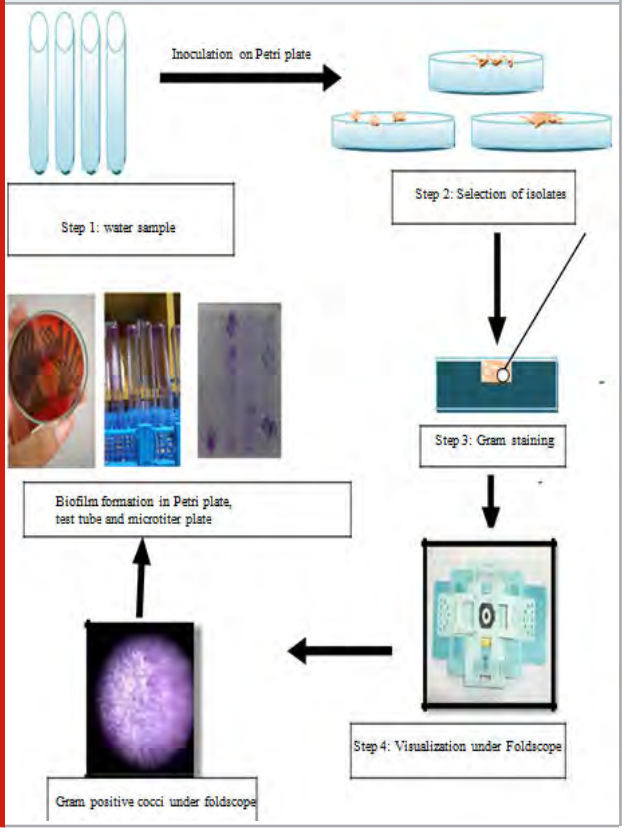
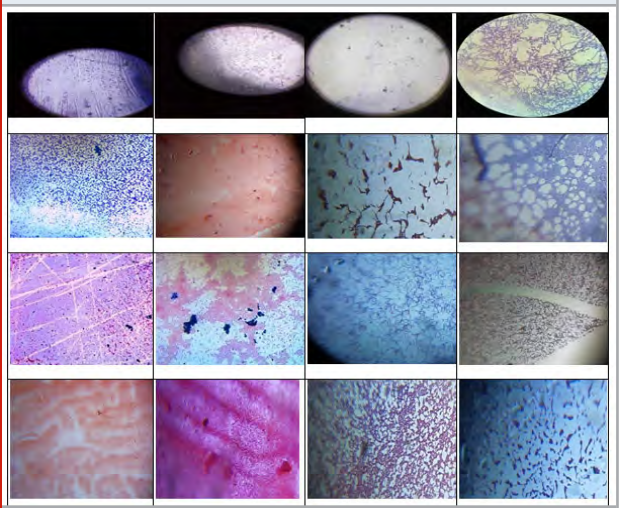


Figure 4c: Gram staining observation of bacteria in water samples through foldscope (Magnification: 140 X)



Analysis of Questionnaires:

The study showed that 47.50 % secondary school students have high level of scientific temper, 36.67 % secondary school students have above average level of scientific temper, 13.33 % have average level of scientific temper, only 2.50 % have below average level of scientific temper and none were having poor level of scientific temper (Fig 5 and Fig 6).

Table 1. Overall students' review on the use of foldscope

Questions	Students' review	
	Rural students (N=300)	Urban students (N=300)
Have you ever used microscope?	50	270
Have you ever prepared slides?	5	35
Have you visited fields for sample collection?	30	150
Have you use microscope in field survey?	0	0
Is Foldscope easy to handle?	280	290
Are the images under foldscope clear?	250	270

Table 2. Overall percentage of scientific temper among secondary school students

Levels	N	% age
High scientific temper	285	47.50 %
Above average scientific temper	220	36.67 %
Average scientific temper	80	13.33 %
Below average scientific temper	15	2.50 %
Poor scientific temper	0	0.00 %
Total	600	100.00%

Figure 5: Overall students' review on the use of foldscope

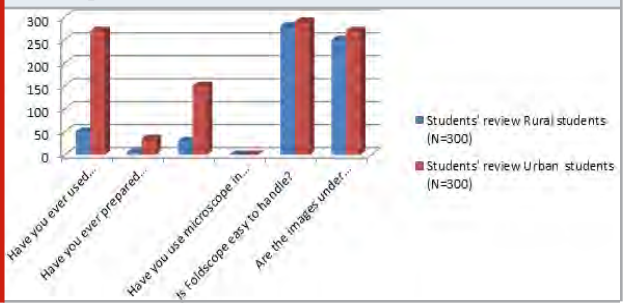
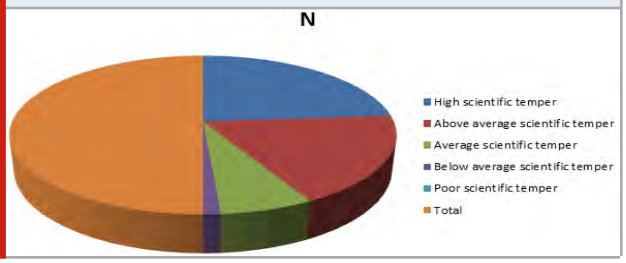


Figure 6: Overall percentage of scientific temper among secondary school students



The table 3 revealed that rural secondary school students show more level of 'High scientific temper' (70.00%) as compared to urban secondary school students (30.00%). It further depicts that about 46.67% of urban secondary school student showed 'Above average scientific temper' while 23.33% of rural secondary students fall at this level. Further, 20.00% of urban secondary students fall at 'Average scientific temper' while only 5.00% of rural student showed 'Average scientific temper'. In rural secondary school, 1.67% of the student showed 'Below average scientific temper' while in urban area, 3.33% of the secondary school student showed 'Below average scientific temper' (Fig 7) (Buragohain et al., 2019).

Table 3. Comparison between rural and urban students on levels of scientific temper

Levels	Rural students		Urban students	
	N	Percentage	N	Percentage
High scientific temper	210	70.00 %	90	30.00 %
Above average scientific temper	70	23.33 %	140	46.67 %
Average scientific temper	15	5.00 %	60	20.00 %
Below average scientific temper	5	1.67 %	10	3.33 %
Poor scientific temper	0	0.00 %	0	0.00 %
Total	300	100.00 %	300	100.00 %

The table 4 showed the mean difference between rural and urban secondary school students on 'Curiosity' dimension of scientific temper scale. The table revealed that there was significant difference between rural and urban secondary school students on curiosity dimension. The calculated t-value 7.634 exceeded the tabulation value at 0.01 level of significance. This justified that the difference between two groups was statistically significant at 0.01 level. Further the mean score of rural (8.95) secondary school students is decidedly better than the mean score of urban (6.80) secondary school students (Thakur et al., 2019).

Figure 7: Comparison between rural and urban students on levels of scientific temper

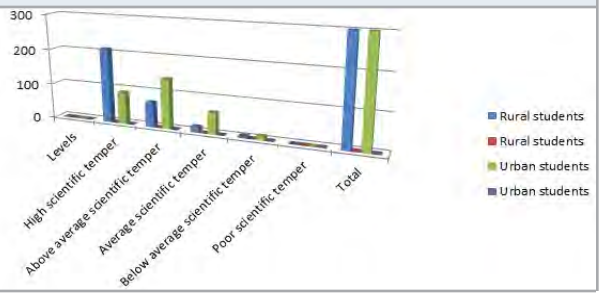


Table 4. Mean comparison between rural and urban students on 'Curiosity' dimension of scientific temper (N=300 each)

Dimension	Group	Mean	Std. Deviation	t-value	Level of Significance
Curiosity	Rural	8.95	1.252	7.634	**
	Urban	6.80	1.925		
**=significant at 0.01					

Table 5. Mean difference between rural and urban students on 'open-mindness' dimension of scientific temper (N=300 each)

Dimension	Group	Mean	Std. Deviation	t-value	Level of Significance
Open-mindness	Rural	7.45	1.535	7.842	**
	Urban	5.65	2.157		
**=significant at 0.01					

The table 5 showed the mean difference between rural and urban secondary school students on 'open-mindness' dimension of scientific temper. The table reveals that there was significant difference between the rural and urban secondary school students on open-mindness dimension of scientific temper. The calculated t-value of 7.842 exceeded the tabulation value at 0.01level of significance. Further, the mean score of rural (7.45) secondary school students was higher than the mean of urban (5.65) secondary school students. (Thakur et al., 2019).

Table 6. Mean difference between rural and urban students on 'objectivity' dimension of scientific temper (N=300 each)

Dimension Group		Mean	Std. Deviation	t-value	Level of Significance
Objectivity	Rural	9.02	1.187	8.450	**
	Urban	7.85	1.970		

**=significant at 0.01

Table 7. Mean difference between rural and urban students on 'Rationality' dimension of scientific temper (N=300 each)

Dimension	Group	Mean	Std. Deviation	t-value	Level of Significance
Rationality	Rural	8.27	1.235	10.485	**
	Urban	6.02	1.910		

**=significant at 0.01

The table 6 showed the mean difference between rural and urban secondary school students on 'Objectivity' dimension of scientific temper. The table revealed that there was significant difference between the rural and urban secondary school students on 'Objectivity' dimension of scientific temper. The calculated t-value of 8.450 exceeded the tabulation value at 0.01level of significance. Further, the mean favored rural (9.02) secondary school student, which implied that rural students are more open to new things than urban secondary school students (Buragohain et al., 2019).

Table 8. Mean difference between rural and urban students on 'Aversion to superstition' dimension of scientific temper (N=300 each)

Dimension	Group	Mean	Std. Deviation	t-value	Level of Significance
Aversion to superstition	Rural	5.85	1.610	5.285	**
	Urban	4.97	2.127		

**=significant at 0.01

The table 7 revealed that there was significant difference between rural and urban secondary school students on 'Rationality' dimension of scientific temper. The calculated t-value of 10.485 exceeds the tabulation value at 0.01level of significance. This justified that the difference between the two groups (rural & urban) was statistically significant at 0.01. The mean of rural (8.27) secondary school students is decidedly more than urban (6.02) secondary school students, which indicated that rural students were more rational, reasonable, and highbrowed than urban (Buragohain et al., 2019).

The table 8 showed the mean difference between rural and urban secondary school students on 'Aversion to superstition' dimension of scientific temper. The table revealed that there was significant difference between the rural and urban secondary school students on this dimension of scientific temper at 0.01level. Further, the mean score favoured the rural (5.85) secondary school students, which implies that rural secondary school students did not follow any belief blindly (Thakur et al., 2019).

Table 9. Mean difference between rural and urban students on 'Overall dimensions' of scientific temper (N=300 each)

Dimension	Group	Mean	Std. Deviation	t-value	Level of Significance
Scientific temper	Rural	39.12	4.017	11.455	**
	Urban	31.85	7.015		

**=significant at 0.01

The table 9 revealed that rural and urban secondary school students differ significantly on composite score of scientific temper scale. The calculated t-value of 11.455 exceeded the tabulation value at 0.01level of significance. This justified that the difference is statistically significant at 0.01. Further, the mean of rural (39.12) secondary school students is better than the mean score of urban (31.85), which indicates that rural secondary school students displayed better scientific temper than urban secondary school students (Thakur et al., 2019).

CONCLUSION

The foldscope instrument is affordably designed to be carried in every student's pocket. Being an effective research tool it helps mainly in the field visits to observe the samples at instant. The smart phones is an another valuable tool to fit with for storing a particular sample image. An interacting relation between the teachers and students about the images of samples was also seen. The foldscope has proved to be a valuable diagnostic tool for the secondary school students that lack space and expenditure. Findings revealed that there was significant difference between rural and urban secondary school students on curiosity.

Rural students are found to be more curious to learn new things and are always ready to go for any adventure trips. Further, the findings depicted that rural and urban secondary school students differ significantly on open-mindness, objectivity, and rationality dimension of scientific temper. Findings shown that rural secondary school students are open to new things. They do not reject any knowledge which conflict with their own idea. Their mind is free from any prejudice and is unbiased. Rural students are inclined to interpret the data prior to the actual observations and experimentation that was made as well as verifies the observation's consistency. The study showed that rural secondary school students were high on 'aversion to superstition' dimension of scientific temper than their counterparts. They rejected the false beliefs and accept scientific facts and explanations.

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Conflict of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Identification of Genetic Relationship Among *Saraca asoca* Genotypes Using Inter Simple Sequence Repeat Markers

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ABSTRACT

Saraca asoca (Roxb.) De Wilde is considered as a medicinally important plant species with higher therapeutic value. It has immense therapeutic value due to presence of various bioactive compounds in it. Geographical locations play important roles in the production of pharmaceutically important compounds and this variation comes from the genetic background of the plants. *S. asoca* is found at different locations of India and may differ in their genetic architecture. Due to high pharmaceutical values it is important to explore diversity among genotypes available in India. The present investigation was carried out to explore inherent diversity at DNA level among 15 *S. asoca* genotypes collected from different geographical locations of India. For this purpose a total of 40 inter simple sequence repeat markers (ISSR) were used. Out of 40 ISSR markers, only 28 markers produced polymorphic bands. The total numbers of bands amplified were 149 out of them 133 bands were found to be polymorphic. *A dendrogram* was generated on the basis of UPGMA clustering. All *S. asoca* genotypes were clustered into two groups and grouping was done according to their collection regions. The study demonstrates higher genetic variability among studied genotypes with the suitability of ISSR markers for phylogenetic analysis of *S. asoca* genotypes. The findings of present investigation shall be helpful in the preparation of strategies for the management of Ashoka. The grouping of genotypes on the basis of their collection areas opens the window to perform future research for variations in the levels of bioactive compounds present in geographically distinct *S. asoca* genotypes of India.

KEY WORDS: GENETIC DIVERSITY, GENOTYPES, DENDROGRAM, ISSR, CONSERVATION, MARKERS.

INTRODUCTION

Saraca asoca (Roxb.) De Wilde, (Family: Caesalpiniaceae), a slow-growing climax forest tree species, is immensely valued for its medicinal properties. *S. asoca* is facing a problem of depletion from its innate habitation in India. It is now categorized as 'vulnerable' and considered 'red listed' by the International Union for Conservation of Nature (IUCN) (Senapati et al. 2012; Mohan et al. 2017). Due to historical evidences *S. asoca*, is generally acknowledged as the 'Ashoka'. This species is found in evergreen forests as well as some other parts of India according to the suitability of climatic conditions.

In terms of therapeutic importance of *S. asoca*, different plant parts such as bark, leaves, flowers and seeds have been found better and these plant parts are currently in use for the production of various medicines (Hegde et al. 2017a). The bark of *S. asoca* has proven its values better among all parts of the plant. It has been used in

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the preparation of such formulations which are generally used to treat gynecological disorders like menorrhagia, bleeding haemorrhoids and disorders associated with the menstrual cycle (Singh et al. 2015). Due to higher therapeutic values the plant is extensively exploited in the legal as well as illegal herbal drug trades (Hegde et al. 2017b; Hegde et al. 2018b; Yadav et al 2019).

Due to the increasing demand of this plant species, the enormous harvesting has pushed the species under 'red listed' category in a short period (Hegde et al. 2018b; Yadav et al 2019). Accurate identification of inherent variability in a rare and endangered plant species is necessary (Nongrum et al. 2012) to save them in their natural environment (Iranjo et al. 2016). Molecular characterization of genotypes of any endangered species collected from wild sources makes a platform to build conservation policies for future deployment of targeted species. Assessment of genetic diversity needs authentic tools which may not be influenced by environmental conditions. Molecular markers are DNA based tools for the analysis of genetic variations within and among the species (Tripathi et al. 2012; Tripathi and Khare, 2016; Hegde et al. 2018a). Hence, the application of molecular markers is important to generate diversity-based data for accurate execution of conservation strategies (Thakur et al 2019).

Molecular markers have also been applied in the field of crop genetics as well as hybridization programmes to transfer targeted gene/s (Kachare et al. 2019; Tiwari et al. 2019). Among all molecular markers inter simple sequence repeats (ISSRs) have proved their ability to characterize various medicinal plant species (Hadipour et al. 2020). These markers are able to generate large amount of precious information on the characterization of plant populations despite having few drawbacks such as reproducibility of bands. However, ISSR markers have been enormously applied to characterize various medicinal plant species due to the easy application and non requirement of prior acquaintance of DNA sequences (Tripathi et al. 2013; Sharma et al. 2016; Hadipour et al. 2020). The genetic diversity analysis among *S. asoca* genotypes collected from different locations including Central India has not been carried out earlier (Hadipour et al. 2020).

Among dominant molecular markers, the superiority of ISSRs has been proved previously in different medicinally important plant species and there are very few reports on the application of ISSR markers in *S. asoca* (Hadipour et al. 2020). So, the objective of the present study was to assess the genetic variability present among collected Indian *S. asoca* genotypes on the basis of ISSR fingerprinting technique.

MATERIAL AND METHODS

Initially an extensive survey was carried out to identify the location of India where *Saraca asoca* found. These locations were considered as potential pockets and geographical parameters (latitude and longitude) of such locations were noted down in the research notebook. For plant material, a total 15 plants of *Saraca asoca* (Table 1) were collected from potential pockets of India to assess genetic variability among them for the future conservation purposes.

Table 1. Details of collection site of *Saraca asoca* genotypes

S. no.	Place of collection	Sample code	State	Latitude	Longitude
1	*Kodaikanal*-1	SA1	Tamilnadu	10° 15' N	77 ° 33' E
2	*Kodaikanal*-2	SA2	Tamilnadu	10° 15' N	77 ° 33' E
3	*Melpallum*	SA3	Tamilnadu	10° 20' N	77 ° 33' E
4	*Palani*	SA4	Tamilnadu	10° 26' N	77 ° 29' E
5	*Satyamagalum*-1	SA5	Tamilnadu	11° 29' N	77 ° 13' E
6	*Satyamagalum*-2	SA6	Tamilnadu	11° 29' N	77 ° 13' E
7	*Satyamagalum*-3	SA7	Tamilnadu	11° 29' N	77 ° 13' E
8	*Satyamagalum*-4	SA8	Tamilnadu	11° 29' N	77 ° 13' E
9	*Munnar road forest area*-1	SA9	Kerala	10° 11' N	77 ° 15' E
10	*Munnar road forest area*-2	SA10	Kerala	10° 11' 11N	77 ° 15' E
11	*Gopal swami hill*	SA11	Karnataka	11.12° 42' N	76 ° 36' E
12	*Borivali forest area*	SA12	Maharashtra	19 ° 14' N	72° 50' E
13	*Vasco*	SA13	Goa	15 ° 24' N	73 ° 50' E
14	Veterinary College, Jabalpur	SA14	Madhya Pradesh	23 ° 06' N	79° 55' E
15	Gwarighat, Jabalpur	SA15	Madhya Pradesh	23 ° 06' N	79° 55' E

For DNA extraction, genomic DNA of each collected plant of *S. asoca* was isolated using protocol standardized by Doyle and Doyle (1990). Extracted DNA was purified to remove the impurities like RNA, proteins and polysaccharides. Purity of DNA was checked by taking the ratio of Optical Density (O.D.) using UV-Spectrophotometer at 260 nm to that of 280 nm. The quality of DNA was also checked by horizontal submarine gel electrophoresis on 0.8% agarose gel. The quantity, quality and integrity of isolated DNA were also checked by gel electrophoresis.

PCR amplification and electrophoresis: For molecular diversity analysis initially a total of 40 ISSR primers were screened to check amplification efficiency with randomly selected four DNA samples as template. Amplification of ISSR fragments was performed in 25 µL volumes containing 30 ng genomic DNA, 10 pmol primer (IDT, India), 200 µM of each dNTP and 1 unit of Taq DNA polymerase (Promega) in PCR buffer supplied (TrisHCl, pH 9.0; 15 mM MgCl2). The amplification reaction consisted of an initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 60 s, annealing at 50 °C for 60 s, and extension at 72 °C for 1 min with final extension at 72 °C for 7 min.

ISSR amplifications were performed in thermal cycler (eppendrof realplex) thermal cycler. The amplified PCR products were visualized through gel electrophoresis (GENETIX GX606C) on 1-5% w/v agarose gels in 1X TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA) using ethidium bromide as the staining dye, for 2.0 h at a constant voltage of 70 V. The agarose gels were visualized and documented under UV light using a gel documentation system with geneview W645SC. Reproducibility of bands produced by each primer was confirmed with twice amplification of same bands with each primer and consequently, the steady and reproducible bands were scored for further data analysis.

Table 2 Details of ISSR primers with banding profile used in the present study

S. no.	Primer	Sequence 5'-3'	TNB	PB	MB	PP
1	UBC 807	AGAGAGAGAGAGAGAGT	7	6	1	85.7
2	UBC 808	AGA GAG AGA GAG AGA GC	6	5	1	83.3
3	UBC 809	AGA GAG AGA GAG AGA GG	7	6	1	85.7
4	UBC 810	GAG AGA GAG AGA GAG AT	7	6	1	85.7
5	UBC 811	GAG AGA GAG AGA GAG AC	5	5	0	100
6	UBC 812	GAG AGA GAG AGA GAG AA	4	4	0	100
7	UBC 814	CTC TCT CTC TCT CTC TA	6	6	0	100
8	UBC 816	CAC ACA CAC ACA CAC AT	6	6	0	100
9	UBC 817	CAC ACA CAC ACA CAC AA	4	4	0	100
10	UBC 818	CAC ACA CAC ACA CAC AG	6	6	0	100
11	UBC 820	GTGTGTGTGTGTGTGTC	5	4	1	80.0
12	UBC 830	TGTGTGTGTGTGTGTGG	4	4	0	100
13	UBC 834	AGAGAGAGAGAGAGAGYT	6	5	1	83.3
14	UBC 840	GAGAGAGAGAGAGAGAYT	5	5	0	100
15	UBC 843	CTCTCTCTCTCTCTCTRA	6	6	0	100
16	UBC 844	CTCTCTCTCTCTCTCTRC	5	5	0	100
17	UBC 851	GTGTGTGTGTGTGTGTYG	6	5	1	83.3
18	UBC 857	ACACACACACACACACYG	6	4	2	66.6
19	UBC 860	TGTGTGTGTGTGTGTGRA	5	5	0	100
20	UBC 862	AGCAGCAGCAGCAGCAGC	4	3	1	75.0
21	UBC 868	GAAGAAGAAGAAGAAGAA	4	3	1	75.0
22	UBC 880	GGAGAGGAGAGGAGA	8	8	0	100
23	UBC 881	GGGTGGGGTGGGGTG	4	4	0	100
24	IUF016	ACTGACTGACTGACTG	4	3	1	75.0
25	IUF017	GACACGACACGACACGACAC	6	6	0	100
26	IUF019	AGAGAGAGAGAGAGAGAGAGG	5	3	2	60.0
27	IUF021	AGAGAGAGAGAGAGAGAGAGC	6	4	2	66.6
28	IUF022	AGAGAGAGAGAGAGAGAGAGT	2	2	0	100
Total			149	133	16	---
Average			5.32	14.75	0.57	89.47

TNB-Total numbers of bands, PB-Polymorphic band, MB-Monomorphic bands, PP-Percentage polymorphism, Y= (C or T), R= (A or G)

Figure 1: Electrophoretic banding pattern of ISSR primer UBC 880 with 15 *S. asoca* genotypes

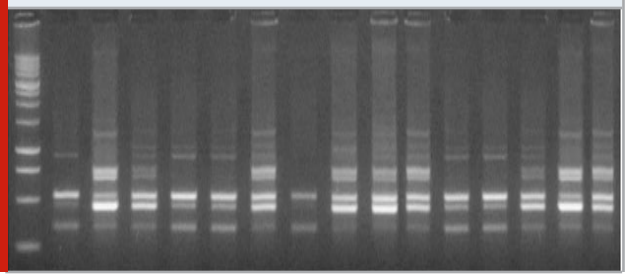
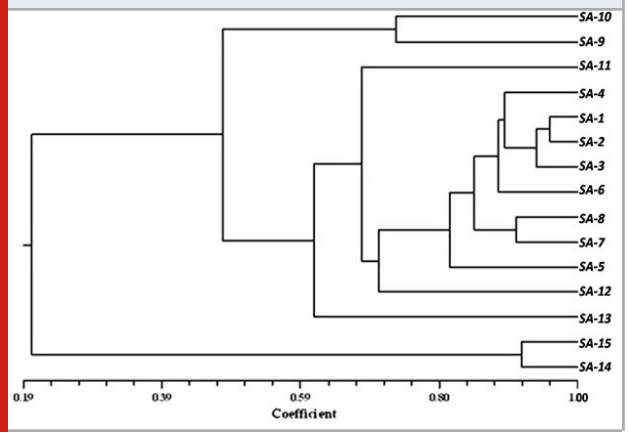


Figure 2: Inter Simple Sequence Repeat markers based dendrogram representing genetic relationship among 15 *Saraca asoca* genotypes



For data analysis, band scoring was performed on the basis of their presence and absence. The presence of band was denoted by '1' while the absence of band was denoted by '0'. After scoring the binary data matrix was prepared using excel sheet. A dendrogram was constructed by using Unweighted Pair Group Method with Arithmetic average (UPGMA) cluster analysis based on the matrix of Jaccard's similarity coefficient with NTSYS-pc (Rohlf, 2000) ver.2.1 to show a representation of genetic relationships among the studied *S. asoca* genotypes.

RESULTS AND DISCUSSION

Inter simple sequence repeat markers technology is one of the easiest and cheaper than other techniques available for DNA fingerprinting. Because of mentioned properties, this technique is also considered to be better than other random fingerprinting methods (Tripathi et al. 2012). Genetic diversity assessment is very imperative for the maintenance of plant genetic resources in their innate habitat. Initially, the amplification of isolated template DNA samples of *S. asoca* plants was performed with 40 ISSR primers (Tripathi et al. 2018; Liu et al. 2020).

Among them only twenty eight primers (Table 2) have been found to be consistent with clear bands and were further selected for the study. Electrophoretic banding pattern of UBC 880 primer is illustrated in fig. 1. During the present study, total 149 bands were amplified and out of these 133 bands were found to be polymorphic and 16 bands were monomorphic. Monomorphic bands are those which are present in all individuals, polymorphic are present in one or more but not all individuals (Mehetre et al. 2004).The average numbers of total band was 5.321 while average numbers of polymorphic bands was 4.75. The number of bands produced per primer ranged from 2 (IUF022) to 8 (UBC 880). Among all studied markers the highest percentage (100%) of polymorphism was demonstrated by fifteen markers however thirteen markers had less polymorphism comparatively. The lowest polymorphism (60%) was demonstrated by marker IUF019. The average percentage of polymorphism was 89.47%. However, only 43% variations have been reported previously by Hegde et al. (2018a) among *S. asoca* genotypes while studying genetic diversity analysis among different populations with the use of ISSR markers (Hegde et al. 2018a).

Cluster analysis was performed on the basis of similarity co-efficient generated from ISSR profiles. The cluster analysis grouped all the *S. asoca* genotypes under study in two groups i.e. group A and group B (Fig. 2). Group A is a major cluster consisting 13 *S. asoca* genotypes. Group A was further divided into two sub groups C and D. Sub group C consisted only two genotypes coded as SA-9 and SA-10. Both of these genotypes were collected from Kerala. Sub group D contained 11 genotypes of *S. asoca*. Among the genotypes clustered together in sub group D a total of 8 genotypes of *S. asoca* collected from different locations of Tamilnadu. Remaining 3 three genotypes in sub group D were from Karnataka, Maharashtra and Goa (Hegde et al. 2018a).

The clustering of genotypes in sub group D demonstrated higher similarity among the genotypes collected from Tamilnadu. However, the genotypes collected from Maharashtra, Goa and Karnataka showed genetic distance from Tamilnadu genotypes and were clustered distantly. Group B contained only two genotypes collected from Jabalpur, Madhya Pradesh. Both of these genotypes had higher resemblance with each other and clustered together. However, these both of the genotypes had high level of genetic dissimilarity with other studied genotypes so, these both were clustered distantly. The grouping of the genotypes demonstrated genetic similarity between the genotypes collected from same geographical locations. Only few previous reports are available on clustering information of *S. asoca* genotypes collected from different locations with the use of ISSR markers. Hegde et al. (2018a) found similar clustering pattern during their study on *S. asoca*. In their report genotypes under cultivation were grouped separately from the individuals of wild genotypes (Hegde et al. 2018a).

The findings of the current investigation revealed the successful utilization of ISSR markers for the assessment of available variability among 15 *S. asoca* genotypes at molecular level. Idrees and Irshad (2014) stated the polymorphism showed by molecular markers may be due to change or mutation in targeted loci or variation

of nucleotide. These alterations are responsible for the presence of genetic variability between and among individuals. In recent studies, ISSR have been applied for the assessment of inherent multiplicity in diverse endangered species medicinal plants (Hamouda 2019; Hadipour et al. 2020).

CONCLUSION

In conclusion, the present study demonstrated the applicability of ISSR markers in molecular variability assessment of 15 Indian *S. asoca* genotypes collected from various locations of the country. ISSR markers were found to be suitable to group the studied genotypes according to their collection sites. *S. asoca* genotypes collected from Kerala as well as Madhya Pradesh showed higher genetic distance from other genotypes. However, genotypes of Tamilnadu state had close similarity and they grouped together. Data generated in the present study will provide a base to develop conservation strategies for medicinally significant *S. asoca* species which has been already pushed into 'red listed' category.

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Conflict of interest: Authors declare no conflict of interest.

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In-Vitro Antibacterial Activity of Black Tea (*Camellia sinensis*) Mediated Zinc Oxide Nanoparticles Against Oral Pathogens

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ABSTRACT

The zinc oxide nanoparticles (ZnO Nps) were synthesized using the aqueous extract of black tea (*Camellia sinensis*) and zinc sulphate as the zinc source by the green synthesis method. This method has numerous advantages such as nontoxic, eco accommodating, less time consuming, ease to scale up for the synthesis of zinc oxide nanoparticles without incorporating any organic chemicals. In this present investigation the black aqueous extract was prepared and zinc oxide nanoparticles were synthesized using constant magnetic stirring. The prepared zinc oxide nanoparticles were purified using centrifugation techniques. The synthesized nanoparticles were characterized using UV-Visible spectroscopy. The UV-Vis range was recorded to screen the formation of the nanoparticles by using black tea aqueous extract, which displayed a blue shifted absorption peak around 350-400 nm. The higher level of phenolic mixes of the black tea extract confirms the reducing activity on the metal oxides. The antibacterial activity of zinc oxide nanoparticles synthesized using black tea extract was analysed using Muller hinton agar well diffusion method. The agar well – diffusion technique was utilized to test the antibacterial activity on chosen oral pathogens such as *Streptococcus mutans, Staphylococcus aureus, Enterococcus feacalis*. The antibacterial activity results show the potential effect of ZnO Nps which depicts equal and effective antibacterial activity when compared to that of synthetic drugs. Based on our results of antibacterial activity of black tea mediated zinc oxide nanoparticles it shows very good oral pathogen in in-vitro and it may be used many dentistry product developments such as tooth paste, mouth wash, dental varnish etc.

KEY WORDS: GREEN SYNTHESIS, BLACK TEA, ZNO NANOPARTICLES, ANTIBACTERIAL ACTIVITY..

INTRODUCTION

Nanomaterials are particles having nanoscale measurement, and nanoparticles are small estimated particles with upgraded synergist reactivity, warm conductivity, non-straight optical execution and compound relentlessness attributable to its enormous surface territory to volume proportion (Tabrez et al., 2016; Rajeshkumar and Bharath 2017; Happy et al., 2018). NPs have begun being considered as nano antibiotics in light of their antimicrobial activities (Sastry et al.,2003; Rajeshkumar and Poonam Naik, 2018). Nanoparticles have been incorporated into different modern, wellbeing, nourishment, feed, space, compound, and beauty care products which requires a green and condition cordial way to deal with their synthesis (Rao and Gautham, 2016; Ponnanikajamideen et al., 2018; Rajeshkumar et al., 2018). Synthesis of metal nanoparticles is dependent

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on their size, shape and dissemination for building up the technology of nano-materials fields. The metallic nanoparticles, for example, gold, silver, iron, zinc and metal oxide nanoparticles indicated interesting in biomedical applications (Bhattacharya and Mukerjee, 2008; El Batal et al., 2008; El Barbary and El-Sawy, 2017; El Batal et al., 2017; Karthiga et al., 2018; Sánchez-López et al., 2020).

Zinc oxide is exceptional material that exhibits semiconducting, piezoelectric, and pyroelectric properties and has differed applications in transparent electronics, ultraviolet (UV) light matters, piezoelectric devices, compound sensors, spin electronics, individual consideration items, coating and paints (Akhtar et al., 2012). Biosynthesis of ZnO NPS from plants, for example, *Aloe vera, Sargassum muticum, Eichhornia crassipes, Borassus* flabellifer fruit, and furthermore in some bacterial and contagious species, for example, *Bacillus subtilis* and *Escherichia coli, Ureolytic microorganisms, Lactobacillus plantarum* have been reported (Vijayakumar et al., 2015; Nikolova and Chavali, 2020). Camellia sinensis is a species of evergreen shrub classified in the family Theaceae found throughout India. Periodontal malady is a genuine bacterial disease, wherein the gums and bones that help the teeth become truly harmed. Tea can be utilized as a characteristic remedy for periodontal ailment (Sánchez-López et al., 2020).

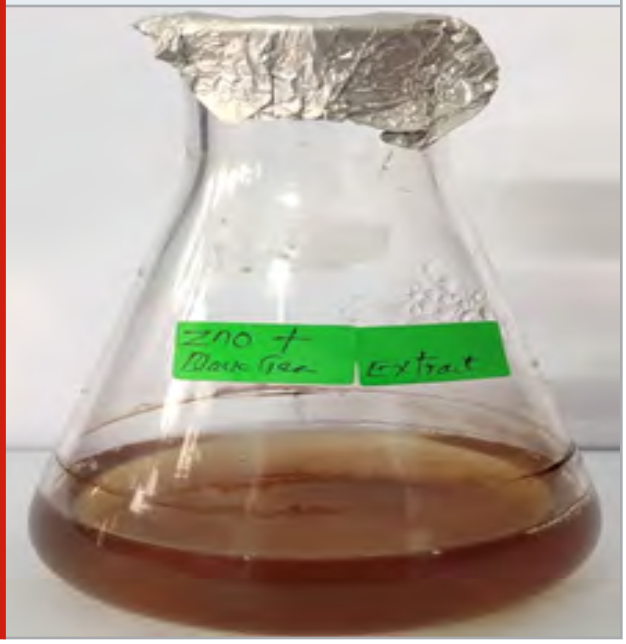
Arbitrary reviews have detailed that Black tea lessens the rate of dental illness (Stefano Petti and Scully, 2009). The antimicrobial activity of black tea (Almajano et al., 2008) is presumably because of their capacity to shape a complex with extra-cellular and solvent proteins, which ties to bacterial cell divider. Progressively lipophilic flavonoids may disrupt microbial membranes (Samy and Gopalakrishnakone, 2010; Rothenberg and Zhang, 2019). In this current investigation, the black tea extract was used as a reducing and stabilizing agent to obtain zinc oxide nanoparticles through green synthesis method. The synthesized nanoparticles were subjected to characterization such as UV-Visible spectroscopy and the antibacterial efficacy of black tea extract mediated zinc oxide nanoparticles was also tested.

MATERIAL AND METHODS

The chemicals used in this study such as Zinc sulphate, Mueller Hinton agar, were purchased from Hi-media laboratories Pvt. Ltd, India. Bacterial cultures such as *Staphylococcus aureus, Streptococcus mutans, Enterococcus faecalis* were isolated and collected from Saveetha dental college and hospital, SIMATS, Poonamallee, Tamilnadu, India. The preparation of plant extract was done using Black tea powder (*Camellia sinensis*) was bought at a supermarket near Poonamallee. To prepare the extract,1g of black tea powder was dissolved in 100ml of distilled water and boiled at 60-80°C for 10 minutes using a heating mantle. The boiled extract was filtered using Whatmann No.1 filter paper. The filtrates were stored in 5°C for further experiments.

The synthesis of Zinc Oxide nanoparticles was done using Black Tea extract. To prepare black tea (*Camellia sinensis*) mediated zinc oxide nanoparticles, 0.2M of zinc sulphate was dissolved in 60ml of distilled water and kept in magnetic stirrer for few minutes. To that 40ml of filtered black tea extract (*Camellia sinensis*) was added. The solution was kept in magnetic stirrer at 650-800rpm for 72 hours. The synthesized black tea extract mediated zinc sulphate nanoparticles were centrifuged at 8000rpm for 10 mins. The obtained pellet was calcined using a hot air oven at 70°C for 2 hours and preserved in air tight vials for further use.

Figure 1: Synthesis of black tea extract (*Camellia sinensis*) mediated Zinc oxide nanoparticles.

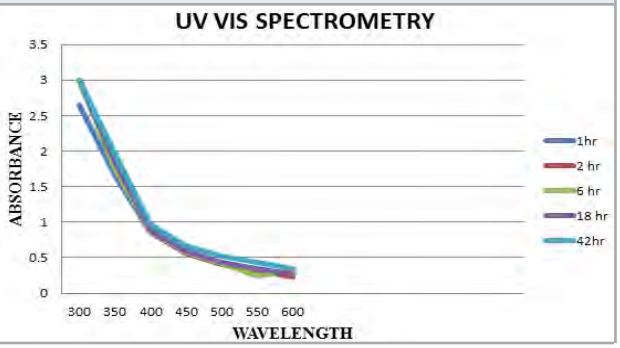


The characterization of Zinc Oxide nanoparticles was done using UV-vis spectrophotometer (uv-2450, Shimadzu) was used to determine the optical property of black tea mediated zinc oxide nanoparticles in the wavelength range of 300-600nm.The determination of antibacterial activity of Zinc Oxide nanoparticles was done by agar well diffusion method. The Mueller Hinton Agar plates were prepared and each plate were swabbed with four different oral pathogens such as *Streptococcus mutans, Staphylococcus aureus, Enterococcus feacalis.* A gel puncher was used to cut four wells on each Petri plates. To the first three wells, black tea extract mediated zinc oxide nanoparticles was added in different concentrations 25µL,50µL,100µL respectively. The effects were compared with that of the standard antibiotic (Amoxicillin) in the concentration of 10µg/mL. The plates were incubated at 37°C for 24hours. The antibacterial activity was determined based on the measurement of the zone of inhibition formed around the well.

RESULTS AND DISCUSSION

Uv-Visible Spectrometry

Figure 2: UV Visible spectra of synthesized black tea mediated zinc oxide nanoparticles.



The colour change reaction from light brown to dark colour in fig 1 preliminarily confirms the reducing and stabilizing activity of black tea extract. Fig 2 shows the surface plasmon resonance of the synthesized nanoparticles which was exhibited in the highly blue-shifted absorption region at 350-400 nm. The black tea mediated zinc oxide nanoparticles shows the maximum absorption peak at 385 nm. Recent studies such as (Sana et al., 2020) correlates with the UV results of black tea mediated zinc oxide nanoparticles.

Antimicrobial Activity

Figure 3: Antibacterial activity of black tea mediated zinc oxide nanoparticles against oral pathogens.

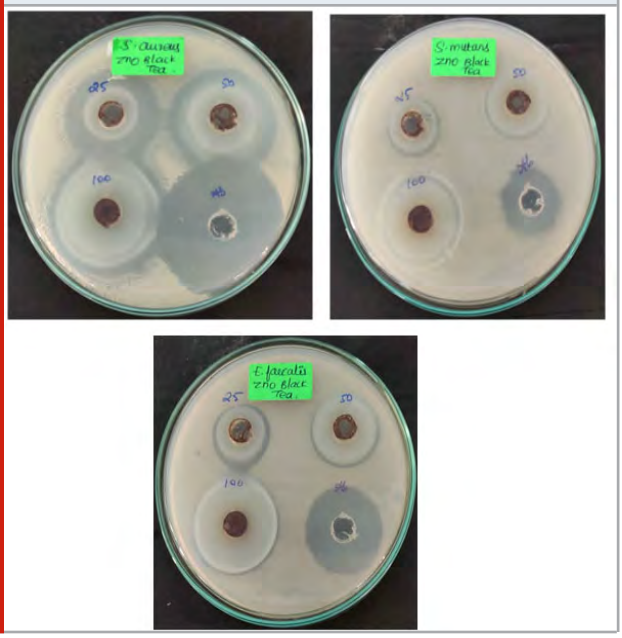
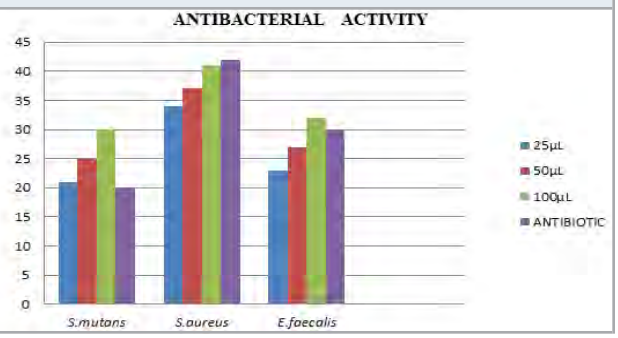


Fig 4 shows that *Staphylococcus aureus* is the most sensitive bacterium followed by *Enterococcus faecalis* and *Streptococcus mutans*. The gram-positive organisms such as Staphylococcus aureus shows its maximum zone of inhibition at 100µL concentration with zone diameter of 41±1mm and that is followed by Streptococcus mutans which shows its maximum zone of inhibition at 100µL concentration with zone diameter of 30±1mm. The gram negative organism *Enterococcus faecalis* shows its maximum zone of inhibition at 100µL concentration with zone diameter of 32±1mm. In this study, gram positive organisms *Staphylococcus aureus, Streptococcus mutans* and gram-negative organism *Enterococcus faecalis* showed resistance to the commercial antibiotic drug which in turn represents the better antibacterial activity of black tea mediated zinc oxide nanoparticles (Ifeanyichukwu et al., 2020).

Figure 4: Histogram of antibacterial activity of Zinc oxide nanoparticles



CONCLUSION

The current study reveals that zinc oxide nanoparticles can be integrated in a simple method utilizing black tea extract. Black tea extract interceded zinc oxide nanoparticles demonstrated phenomenal antibacterial activity against oral pathogens. The investigated biosynthetic arrangement of zinc oxide nanoparticles has intense applications in biomedical and biotechnological applications with various favourable circumstances, for example, cost adequacy and pharmaceutical applications just as for huge scope business creations. Further research on this examination relegate potential applications in dental field which will be proficient to fix dental related illnesses.

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Fusidic Acid as a Potential Drug Candidate Against Infectious Gram Positive Bacteria

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ABSTRACT

Bacterial pathogens have developed multidrug resistance which is a major challenge to global health. Fusidic acid (FA) is a steroid antibiotic commonly used against gram positive *Staphylococcus aureus* (*S. aureus*) infections in clinics. It binds to Elongation Factor-G (EF-G) and inhibits translation by stalling EF-G on ribosome after GTP hydrolysis. Disease causing infectious gram positive bacteria such as Mycobacterium tuberculosis (M. tuberculosis), *Mycobacterium leprae* (*M.leprae*) and *Bacillus anthracis* (*B. anthracis*) pose major health challenge. In order to investigate if FA can be repurposed against these bacterial pathogens, we carried out multiple sequence alignment of EF-G from these bacterial species followed by prediction of their three dimensional structure using homology modeling. These predicted dimensional structures after validation were used for structural analysis with crystal structure of EF-G from *Thermus thermophilus* (*T.thermophilus*) and *S.aureus*. Molecular docking was performed to dock FA into its putative binding site of the predicted three dimensional structures of these bacterial EF-G.Multiple sequence alignment of EF-G sequences from these bacteria showed sufficient sequence identity. Homology models of EF-G from these bacteria were compared with available crystal structure of EF-G from *Thermus thermophilus* (*T.thermophilus*) *Staphylococcus aureus* (*S. aureus*) which revealed overall conserved tertiary structure. Docking of FA to homology models suggested that the antibiotic in principle bind to EF-G from these gram positive infectious bacteria. We therefore propose that fusidic acid as a strong potential drug candidate against infections caused by these gram positive bacteria.

KEY WORDS: FUSIDIC ACID, ELONGATION FACTOR–G (EF-G), GRAM POSITIVE BACTERIA, INFECTIOUS DISEASES.

INTRODUCTION

Fusidic acid (FA) is a steroid antibiotic derived from fungus *Fusidium coccineum* and is commonly used against infections caused by gram positive bacterium *Staphylococcus aureus* (*S.aureus*) (Verbist, 1990). It has an unusual spectrum of activity that includes corynebacteria, nocardia, anaerobes, and Neisseria species, but is used almost exclusively as an anti-staphylococcal agent (Collignon, and Turnidge,1999.). FA inhibits protein synthesis by acting directly on elongation factor-G (EF-G) (Bodley et al., 1969). EF-G is a GTPase which catalyzes the translocation step during elongation phase of protein synthesis. EF-G in complex with GTP translocates the peptidyl-tRNA from A-site to P-site along with GTP hydrolysis. After GTP hydrolysis, EF-G•GDP dissociates from ribosome (Rodnina et al.1999). In the presence of fusidic acid, EF-G remains bound to the ribosome after GTP hydrolysis, sterically blocking the next stage in protein synthesis. (Burns et al., 1974; , Willi et al., 1975 Belardinelli and Rodnina, 2017).

Resistance to fusidic acid is primarily caused by mutations in fusA gene which encodes EF-G and several mutants

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conferring FA resistance in EF-G from *Salmonella typhimurium* [*S. typhimurium*) and from *S. aureus* were identified and phenotypically characterized in vivo (Johanson and Hughes, 1994; Laurberg et al.,2000).. Crystal structures of EF-G have been determined from *Thermus thermophilus* (*T. thermophilus*) and *S.aureus* (Ævarsson,et al.,1994; Chen et al., 2010; Czworkowski et al.,1994; Laurberg et al., 2000;). The protein is an elongated molecule composed of five domains (I-V) whose overall shape resembles that of a tadpole. Domain I (G domain) is the nucleotide binding domain. Domain I and II constitute a single globular unit while domain III, IV and V constitute another block. The domain interface (domain G/ III/ V) has been proposed to undergo conformational change implicating conformational dynamics of the molecule (Lin et al., 2015; Stark et al.,2000 Waterhouse et al 2018).

Availability of the structure of EF-G allowed to map FA resistant mutants as well as to provide possible explanation for their mechanism of action and for the probable binding site of FA. Main conclusions were a) resistant mutants are spread all over the EF-G indicating that few mutants might be directly interacting with FA binding site. b) Three prominent clusters of mutants were identified mapping to G domain, domain III and domain V. c) Most likely these mutations in clusters shall restrict the conformational dynamics of EF-G required for its function or shall modulate affinity of EF-G for ribosome/ FA or both. d).The domain interface is the most likely binding site for fusidic acid. Among bacterial pathogens, *M.leprae, M. tuberculosis* and *B. anthrasis* have posed serious health challenges globally, (Katale et al 2020).

In this report, we have addressed the following issues: a) To what extent EF-G from these select bacteria display sequence identity to EF-G from *T. thermophilus* and *S.aureus* so as to permit homology modeling. b) Whether the tertiary structure is conserved among EF-G homology models when compared with EF-G crystal structure and c) If fusidic acid can bind to proposed putative binding site in these modeled structures. We report that there exists significant overall sequence identity among EF-G sequences from these bacteria to allow homology modeling. The overall structural similarity of these models is quite good when compared with EF-G structure from *T. thermophilusand S.aureus*. The domain interface, the putative binding site for fusidic acid is quite conserved at sequence as well at structure level. Docking of fusidic acid to these models demonstrated that domain interface is the only cavity, where fusidic acid may bind. These results strongly suggest that fusidic acid as a potential drug candidate against these pathogenic bacteria.

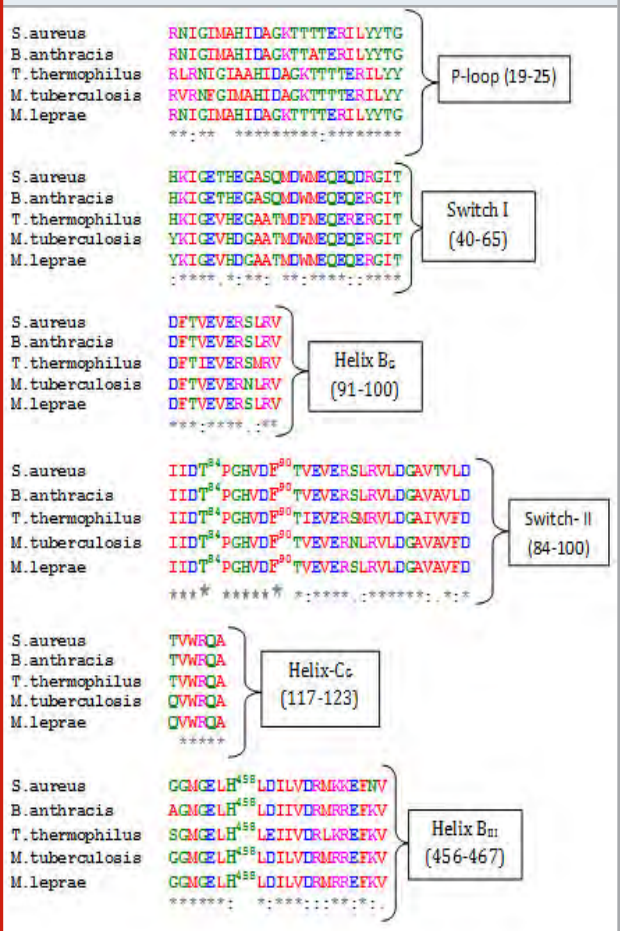
MATERIAL AND METHODS

Sequence alignment: EF-G sequences of select infectious gram positive bacteria were retrieved from the SWISS PROT (Table 1.). Multiple sequence alignment of the sequences was performed using CLUSTALW with default parameters (Larkin et al., 2007).

Homology modeling: The three dimensional structure of the EF-G from the select bacteria were modeled using the SWISS MODEL (Waterhouse et al., 2018). Stereochemical quality of the homology models were validated through PROCHECK (Laskowski et al.,2001) Visualization of the models and analysis was performed using PyMOL and figures were also generated through PyMOL.

Molecular Docking: Fusidic acid was docked with homology models using Patchdock (Schneidman-Duhovny et al., 2005). Patchdock is geometry- based molecular docking algorithm which evaluates molecular shape complementarity between macromolecule and ligand.

Figure 1: Multiple Sequence Alignment of EF-G sequences. Identical residues are marked (*) and similar with (:). Bold and numbered residues shows few select residues of domain interface.



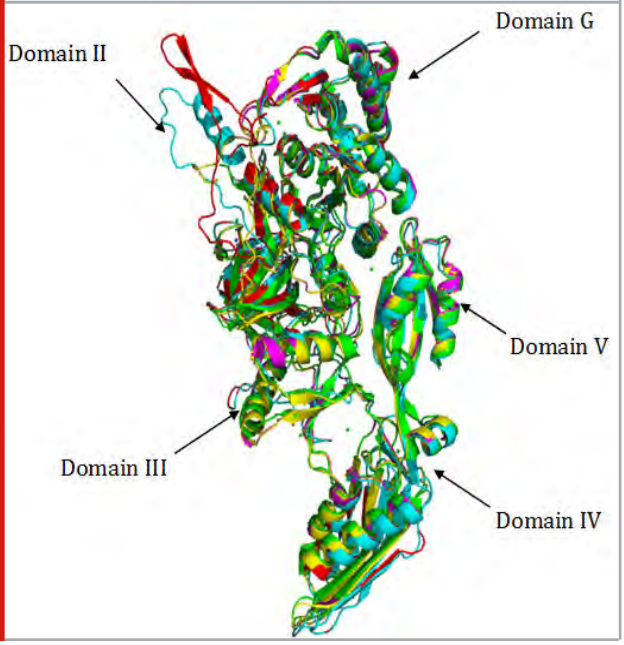
RESULTS AND DISCUSSION

Elongation factor-G belongs to the GTPase superfamily of proteins. The members of this superfamily shares a nucleotide binding domain and two structural dynamic regions, called switch I, switch II, along with phosphate – binding loop (P- loop) which forms the part of nucleotide binding site (Bourne et al., 1991). The protein is composed

of five domains and can be thought of two structural blocks, block 1(domain I and II) and block II (domains III – V). Block II can rotate with regard to block I (Lin et al., 2015). Alignment of EF-G sequences from these select gram positive bacteria reveals significant conservation of consensus elements of GTP binding proteins like P-loop (19-25), switch-I (40-65), switch II (84 -100). Also, secondary structural elements which are part of interface between domain G, domain III and domain V, like helix BG [91-100), helix CG (117-123) and helix BIII (456-467) are also quite conserved (Fig. 1).

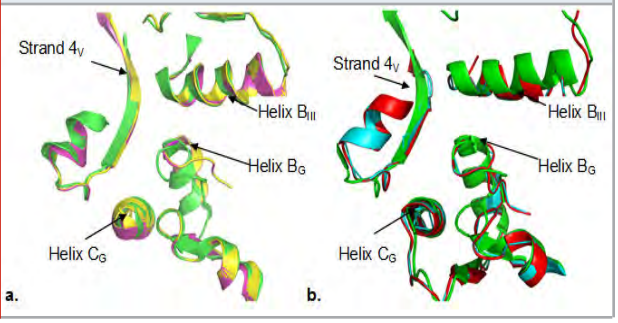
Pairwise alignment of each of the EF-G sequence from these bacteria with that of *T. thermophilus* reveals significant overall sequence identity, 62 % with *S. aureus*, 61% with *M. tuberculosis*, 67% with B. anthracis and 60% with M. leprae (data not shown). Significant sequence overall identity of these bacterial sequences with EF-G from *T. thermophilus* and *S. aureus* allowed structure prediction of these bacterial EF-G using homology modeling. All the models were evaluated for their stereo-chemical quality. The homology models displayed satisfactory Ramachandran plot statistics (data not shown).The overall fold of the modeled structures are very similar to the elongated shape of the EF-G crystal structure (Fig. 2).

Figure 2: Superimposition of C_α traces of crystal structure of EF-G from *T. thermophilus* (green), *S. aureus* (magenta) with homology models of EF-G from B.anthracis (yellow), *M. tuberculosis* (cyan) and *M.leprae* (red).



Superimposition of C_α backbone coordinates of EF-G. from *T. thermophilus* with homology models showed root – mean - square deviations (rmsd's) of 1.24 Å in case of *M. tuberculosis*, 1.29 in case of *M. leprae* and 0.72 Å in case of *B. anthracis* respectively. The crystal structure of EF-G from *T. thermpophilus* when superimposed with crystal structure of *S.aureus* (PDB 2xex) showed rmsd of 0.71 A.This reflects quite conservation of the overall fold of the protein in case of EF-G from these gram positive bacteria suggesting strong conservation of the tertiary structure. It has been proposed that the probable binding site of fusidic acid is domain interface (Belardinelli and Rodnina 2017). Some of the key residues located at this interface are Thr84, Phe90, His458, Asp435, Thr437, G453, which are highly conserved in all the sequences (Fig.1).A close inspection of the domain interface of the models revealed, the putative binding pocket is also significantly conserved at structural level. The important elements of this interface are helix BG and helix CG from G domain, helix BIII and strand 4V from domain III and domain V respectively (Fig.3).

Figure 3: Zoom in view of the superimposition of the backbone coordinates of domain interface (domain G/ III/V) a). *T.thermophilus* (green) with *B.anthracis* (yellow), and *S.aureus* (magenta) b) *T. thermophilus* (green) with *M.tuberculosis* (cyan) and *M.leprae* (red). Secondary structure elements, helix BG, helix CG, helix BIII and strand4V are labeled.



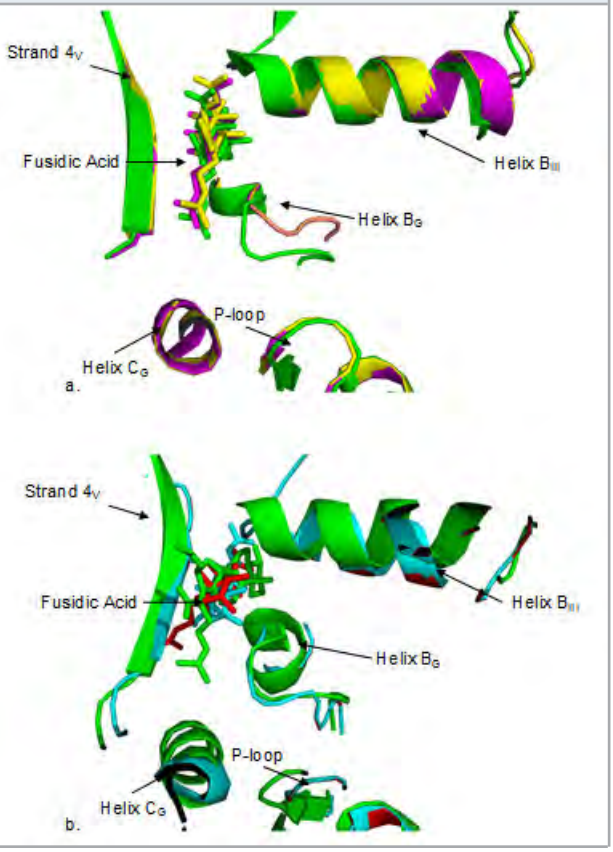
Spontaneous mutations observed under fusidic acid selective pressure are mainly clustered in helix BG and helix CG. The domain interface is structurally well conserved both in case of B. anthracis and *S. aureus* (Fig. 3a) as well as in case of *M. tuberculosis* and *M. leprae* (Fig.3b). Many of the domain interface residues (Phe90, His458, Asp435, Thr437, G453) in fact are known to confer strong resistance to fusidic acid upon mutation (Hansson et al., 2005). Structural aspects of fusidic acid resistance and sensitivity have emphasized the role of domain G/ domain III/domain V interface as a key component of the FA binding site, (Hansson et al., 2005).

In order to investigate the domain interface as most probable binding cavity for fusidic acid, docking of fusidic acid with all homology models as well as with the crystal structure of EF-G from *T. thermophilus* and *S. aureus* was performed with Patchdock, a geometry based molecular docking algorithm. This algorithm focus on local shape feature matching. The sequential three major stages in the algorithm are a) molecular shape representation of the target and the ligand b) surface patch matching of the target and ligand c) Filtering and scoring of candidate complexes from second stage. Solutions with significant unacceptable steric clashes between target and ligand atoms are filtered out and discarded. Solutions with minor clashes (reflects conformational change of the

molecular surface upon docking of the ligand) are scored based on geometric compatibility based on the size of the computed interface.

Thus solutions are ranked based on geometry scores, interface area size and desolvation. The top twenty solutions were analyzed and the best solution among them based on above mentioned scores as well as visual inspection was selected in each case. In order to evaluate the binding position, domain interface of each EF-G model was compared with the docked structure of crystal structure of EF-G from T. thermophilus by superimposition of protein C_α backbone coordinates. Inspection of the domain interface of docked structures of EF-G from *T. thermophilus*, along with *S.aureus* and *B. anthracis*, showed quite similar position and orientation of fusidic acid in the putative binding site.

Figure 4: Close up view of domain interface of EF-G structures docked with fusidic acid. Docked FA is shown in the same color as the color code of the protein. a) *T. thermophilus* (green) with *B. anthracis* (yellow), *S.aureus* (magenta) b) *T. thermophilus* with *M.tuberculosis* (cyan) and *M.leprae* (red)



FA is docked in quite similar position as well as orientation in a) while the position seems to be similar in b), orientation is different in each case. Both in a) and b), FA is in close proximity to helix BG, helix BIII and strand 4V. Figure 4b shows the results of superimposition at domain interface when docked structure of EF-G from *T. thermophilus* is compared with that of *M. tuberculosis* and *M. leprae*. FA seems to bind almost in similar position in the interface as in case with *S.aureus* and *B. anthracis* (Fig. 4a), yet the orientation seems to be quite different. It is known that FA binds to EF-G only when it is bound to ribosome (Belardinelli and Rodnina 2017).

Fusidic acid does not inhibits GTP hydrolysis and translocation by EF-G but prevents the dissociation of EF-G• GDP from ribosome binding (Borg et al., 2015). Nonetheless, structural mapping of fusidic acid resistant mutants in *S. aureus* and *S. typhimurium* led to proposal of a putative binding site for fusidic acid, implicating the interface of domain G with domain II and domain V as the most probable binding site (Laurbert et al., 2000) The current hypothesis is that that the domain interface is the most likely binding site for fusidic acid, especially implicating the role of Phe 90 in binding of FA to its putative site (Koripella et al., 2012). It is also interesing to note that some strong FA resistant mutations are located in the domain interface (Nagaev et al., 2001). Among these residues, Thr 84, Phe 90 are part of a loop which comprises switch II preceding helix BG . His 458 is part of helix BIII and G453 is part of domain interface.

Fusidic acid has been shown to be effective in vitro against certain clinical isolates of *M.tuberculosis* [Hoffner et al., 1990; Cicek-Saydam et al., 2001). Also strains of *M. tuberculosis* mono- or multiresistant to standard antituberculosis drugs have been reported to be susceptible to fusidic acid (Fuursted et al., 1992). It has been demonstrated that FA has excellent pharmacokinetics and in vitro activity against extracellular and intracellular *M. leprae* (Franzblau et al., 1992). There is only one reported clinical trial of FA for lepromatous leprosy [Franzblau et al., 1994). Little FA resistance has been reported against *B. anthracis* isolates [Odendaal et al., 1991). The results presented in this paper demonstrate significant conservation of EF-G sequence as well as structure along with strong conservation of domain interface among these gram positive pathogenic bacteria.

These findings strongly suggests that FA shall not be exclusively used as anti-staphylococcal and has a potential to be used as a drug against these gram positive bacterial pathogens.

CONCLUSION

In conclusion, this study demonstrates that fusidic acid, an important antibiotic primary used against *S. aureus* can be repurposed for other gram positive infectious bacteria namely *B.anthracis, M. leprae*, and *M. tuberculosis.*

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Conflict of interest: The authors declare no conflict of interest.

Contributions: RS conceived the work and PK carried out the work under guidance of RS. RS and PK analyzed the results. PK and RS wrote the manuscript. RS finalized the manuscript.

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The Effect of Saline, Drought, and Presowing Salt Stress on Nitrate Reductase Activity in Varieties of *Eleusine coracana* (Gaertn)

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ABSTRACT

This study investigated the specific changes in nitrate reductase (NR) activity through a range of experiments with different salt treatments. Even under desirable normal conditions, higher plants reply on nitrate as the main source of nitrogen for obtaining optimal growth. Although plants can use various forms of nitrogen, its reduced form, ammonia, is preferable for plant growth as it is directly incorporable into organic compounds. This reduction is made possible via a series of catalysis reactions in which two enzymes, including nitrogenase and nitrite reductase, play the main roles in the conversion of nitrate to ammonia. The observations indicated that low-applied NaCl concentrations triggered higher enzyme activity in both Dapoli 3 and HR374 varieties; a low concentration could be described as about 8mM in both the varieties and, specifically, about 40% in HR374. Despite the observed indirect relation between NaCl concentrations in varieties and enzyme activity levels, an increase in NaCl concentration also had a negative (inhibitory) impact on enzyme activity, as it respectively declined by 50% and 40% in Dapoli 3 HR374 after applying 150 mM of NaCl by irrigation. The study presoaked the seeds in different NaCl concentrations before germination and applied a stage of irrigation with three different concentrations of NaCl (8, 3, and 80 mM). The observations indicated that enzyme activity (EA) slightly improved in the extract collected from the leaves. Also, the study did not detect a significant association between a short-time drought and EA; however, as the drought duration increased to eight days, Line2542 and Line958 respectively represented a 45% and 36% EA decline.

KEY WORDS: ELEUSINE CORACANA, NITRATE REDUCTASE, SALINE, DROUGHT, PRESOWING SALT STRESS.

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INTRODUCTION

The catalysis reaction leading to the reduction of N03 to NH_4^+ includes two stages in which the two enzymes mentioned above are involved. Two electrons allow for reducing NO_3^- to NO_2^- in the first stage by

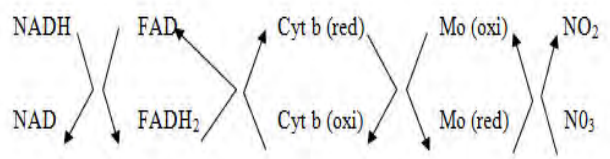
$$NR{:}NO_3 + 2H + + 2e \text{ ------> } N02 + H_2O.$$

Having positive effects on plant growth, NR is also regarded as the leading enzyme of the pathway, involved in the incorporation of nitrate to amino acids (Campbell 1985, Gholamin and Khayatnezhad 2012). Many research

studies report a significant positive correlation between NR activity and plant growth (Zieserl, Rivenbark et al. 1963). With prosthetic groups containing flavin adenine dinucleotide (FAD), haem, and molybdenum, nitrate reductase is considered as an intricate protein, with a large structure and a heavy molecular weight, which consists of two subunit proteins each near 115 kD (Gholamin and Khayatnezhad 2020).

Evans and Nason (Evans and Nason 1953), for the first time, discovered NR cotyledons of some soybean varieties, and ever since, other research studies have detected this enzyme in all parts of the plant (Gholamin and Khayatnezhad 2020). Research concerning nitrate reductase faces two challenging issues regarding the NR localiza¬tion and regulation, as based on the results of many studies, a few researchers speculate it is detectable in the cytoplasmic space (Suzuki, Gadal et al. 1981). Vaughn et al. (Vaughn, Duke et al. 1984) examined the fixed tissue selection of soybean cotyledons in a series of immunofluorescence studies and confirmed the cytoplasmic localization of NR. Yet, NR is present in chloroplasts based on the results of relevant research confirm (Losada and Guerrero 1979, Gholamin and Khayatnezhad 2020, Gholamin and Khayatnezhad 2020).

K¬amachi et al. 1987 conducted an immunogold localization study recently, examining spinach chloroplasts and they concluded that NR is specific to chloroplasts (Kamachi, Amemiya et al. 1987). On the other hand, some research has provided evidence suggesting plastids in roots are the points where the NR is localized (Layzell 1990). Proposedly, NR has a two-site ping pong model the reaction mechanism (Campbell 1985, Khayatnezhad 2012, Khayatnezhad and Gholamin 2012). The first acceptor, FAD, receives the two electrons donated by the reduced pyridine nucleotide, which pass through cyt b557 site to Mo cofactor site, where they are donated to NO_3, which gets reduced to NO_2.



Research studies commonly describe NADH as the agent responsible for reducing the activity of NR (Oaks, Wallace et al. 1972). The results of two research studies and suggest that the function of NR is also associable with NADPH FMNH2, and FADH2, and reduced benzyl and methyl viologens (Prakash, Singh et al. 1984).Alterations in the physiological properties and the environmental conditions can effectively adjust NR activity and impact the production and degradation in higher plants (Guerrero, Vega et al. 1981, Khayatnezhad and Gholamin 2012), some of which include changes in light conditions, sources of nitrogen and energy, vital mineral substances, water-induced stress, temperature fluctuations, PGRs, plant age and metabol¬ic inhibitors (Naik, Abrol et al. 1982). Supply of substrat¬e, coenzymes, and energy are the main determinants of NR activity.Nitrate reductase is highly responsive to substrate (Somers, Kuo et al. 1983) with potential quick turnovers (Oaks, Wallace et al. 1972). The balance between enzyme production and turnover is generally an important maintaining factor of NR levels (Lee and Stewart 1979). May research studies have confirmed the necessity of NO_3 as a substrate for NR induction (Rajasekhar, Gowri et al. 1988, Khayatnezhad and Gholamin 2020).

Also, alterations in light conditions are extremely influential on NR induction (Kapoor, Prakash et al. 1987). The observations in relevant research identify two other agents for regulating NR activity, including NH4 and amino acids (Guerrero, Vega et al. 1981), as the end product of NO_3 reduction (NH4 +) is a presumed suppressor of NR activity. Conversely, some scholars associate NH4+ with the stimulation of NR activity (Smith and Thompson 1971, Khayatnezhad and Gholamin 2020). Since NR is a molybdoflavo protein, the presence of Mo, as an essential NR activity stimulating factor is considered universal. As Mo is found in the structure of the NR molecule, plants with Mo deficiency represent lower NR activity compared to those provided with external sources of Mo, in which Nr activity quickly increases (Afridi and Hewitt 1964).

It has been confirmed that saline soil conditions impair the nitrogen assimilation process (Demboski 1999). Based on the results of relevant research indicate that nitrate reduction is believed to be highly affected by external stress conditions. The research by Smirnoff et al., (Smirnoff, Winslow et al. 1985) proposed two main influential factors on NR activity: nitrate availability or protein synthesis, stating any factor affecting those can also influence NR activity. Various factors are proved influential on NR activity, including soil wet content, salinity, and bacterial nitrification.

The findings of a vast range of research represent that medium saline content is a suppressor of NR activity (Lal and Bhardwaj 1987). Rakova et al. (Rakova, Klyshev et al. 1978) studied the effects of salinity on NR activity and structure, finding that salinity conditions cause structural disintegration of this enzyme's molecules; two main factors are involved for this disintegration, including the dissociation of FAD and Mo from the apoprotein respectively in leaves and roots. Furthermore, many other relevant studies confirm the positive association between salinity and increased NR activity. For instance, the study by Boucaud (BOUCAUD 1972) identified salinity as an indirect factor for increasing NR activity, claiming the effect originates from the modification of CO2 assimilation and protein synthesis by salt.

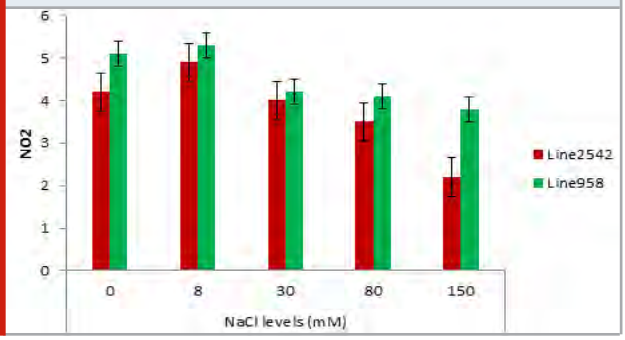
Another relevant study attributed increased NR activity under salinity conditions to higher osmotic potential enhanced by salinity, describing NR activity as specific to different saline conditions (treatments). They found that while a lower $NaHCO_3$ concentration had positive effects, a higher concentration limited the NR activity.

In a similar study, Sharma and Purohit (1986) discovered that NaCl and NaF had a limiting effect on NR activity Joshi (Joshi 1987), though, observed the stimulating effect of NaCl and the inhibitory effect of Na_2SO_4.

MATERIAL AND METHODS

Extraction and Assay buffer: 0.1M Tris HC1 (pH 7.5'), $KN0_3$ 0.1M, Sulphanilamide 1% in 1 N HCl, 1N (1 naphthyl ethylenediamine dihydrochloride 0.1%. In nitrate-to-nitrite reduction reaction, NR plays the accelerating role (catalyst). The study used sulphanilamide as an amino compound coupling with NEDD in the presence of NO2 to synthesize an azo dye. The initial NO_2 concentration corresponds with the amount of azo dye. The study arranged two groups of control and treatment cultivars (Dapoli 3 and HR374) and harvested and chopped the leaves and used their known weight as an enzyme source. The study utilized the method proposed by Klepper et al. (1971) to assay the in vivo NR activity. The amount of assay mixture in the experiment was firstly 2.0 ml containing 0.1M Tris- HCL buffer pH 7.5, 0.005 M KNO_3, and 50 mg of fresh plant material. The experiment put the assay mixture in an incubator for 20 minutes at 370C and terminated the reaction using 1.0 ml of sulphanilamide as an additive after adding NEDD and measured the wavelength of the resulting pink color at 525nm. Finally, the study used the nitrite standard curve to estimate nitrite amounts. The unit of expressing EA was: mg NO_2-/hour/gm fresh tissue.

Figure 1: Interaction NACL levels on Nitrate activity

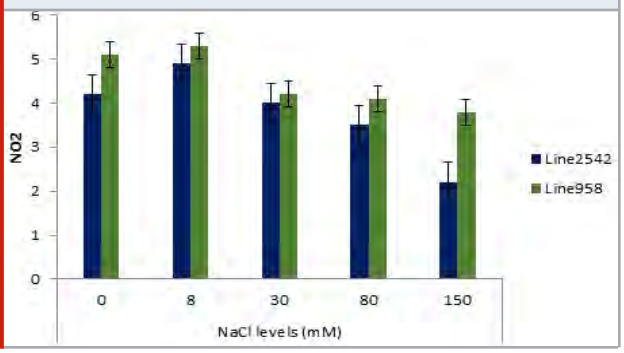


RESULTS AND DISCUSSION

Figure 1 illustrates the data concerning NR activity levels in E. coracana leaves under a treatment regiment with varying NaCl concentrations of sodium chloride. As clearly presented in Fig.1, a low (i.e. 8mM) NaCl concentration results in a higher EA in both varieties of in the study. On the other hand, though, this observation also indicated that increasing NaCl concentration will harm EZ, as it was observed EA respectively declined by 50% and 40% in Dapoli 3 and HR374 under a regiment of 150mM of NaCl. When the study presoaked the seeds in different NaCl concentrations before germination and applied a stage of irrigation with three different concentrations of NaCl (8, 3, and 80 mM). The observations indicated that enzyme activity (EA) slightly improved in the extract taken from the leaves. As depicted in Fig.2, however, an increase in the NaCl concentration to 150mM led to a 25% decrease in EA, representing the relevantly positive effect of a presowing stage on the increasing plants' tolerance to salinity.

Figure 2 illustrates a summary of NR activity under the effect of drought in Line2542 and Line958, indicating non-significance of short-time drought stress; however, the NR activity in Line2542 and Line958 respectively declined by 45% and 36% following an eight-day drought stress application.

Figure 2: Effect of Pre Sowing salt on Nitrate activity



Observations indicate the although a direct increasing role is not attributable to NaCl in terms of NR induction and activity, its ability to modify CO_2 assimilation and protein synthesis can indirectly result in the mentioned effects. In two similar studies, over a decade, on Salsola foetida, Austwenfeld (Austenfeld 1974) found a positive association between low NaCl concentration levels and NR activity. Moreover, they found that alterations in NR activity might have caused fluctuations in the protein content since NR is known for limiting the rate in the whole nitrate assimilation. Joshi, in a study concerning *Cajanus cajan*, found that a similar association between a gradual increase in NR activity and the increasing NaCl concentrations in the soil, attributing the increased activity to the increased osmotic potential as a result of higher NaCl concentrations (Joshi 1984). Several studies, though, claim that salinity can limit NR activity (Sankhla and Huber 1975), suggesting the disintegration of NR under saline conditions in roots of pea and rice (Rakova, Klyshev et al. 1978).

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Creativity and Innovation in Higher Education: A Palestinian Academic Perspective

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ABSTRACT

There is an increasing awareness of the importance of fostering creativity and innovation in higher education due to higher education's critical role in individuals' information age. The study relied on in-depth interviews with 20 faculty professors at MOU University in the West Bank, Palestine, to explore their creativity and innovation perspectives and its importance in higher education. The results indicated that all professors considered creativity and innovation essential in higher education. It gives students opportunities to produce new knowledge and think differently for the benefits of themselves and their communities. The professors presented different factors that encourage creativity and innovation, remove specific regulations and instructions, and provide instructors with the freedom to enhance creativity and innovation in a higher education institution. The study asserted what has been emphasized in the literature on the importance of the university's role in preparing students for industry demands. The study also confirmed on the important role university instructors' role in cultivating and promoting creativity and innovation among the students The study participants confirmed that although augmenting creativity and innovation faces challenges and difficulties in conflict and crisis settings, it is a resilience tool encourage young generation to think creatively and adapt living under difficult situations. It recommended that building partner relationships between universities and the private sector would enhance that role.

KEY WORDS: ACADEMICS, CREATIVITY, HIGHER EDUCATION, INNOVATION, PALESTINE.

INTRODUCTION

An increasing rate of changes, uncertainties, challenges, and problems characterizes today's world. It is a time of complexity, disorder, ambiguity. The labor market is increasingly competitive, demanding employees who can successfully meet the workplace challenges, innovate, act quickly, and present practical solutions to unexpected problems. Under this complexity, creativity and innovation have become the melody, which politicians, business people, employees, teachers, professors, students, and others chant for. The awareness of creativity and innovation's relevance has led several countries' governments to adopt initiatives to implement educational policies that ensure innovation and creativity through education. Scholars from various fields highlighted the need for great attention to developing the creative and innovative capacity across the multiple levels of education, especially in higher education (Donnelly & Barrett, 2008; Gaspar & Mabic, 2015 Alencar & Pereira, 2017).

They are a potential answer to a wide range of problems like social, economic, and educational. They can direct young people love to self-employment, and desire to take positive risks and work hard to make new ideas

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succeed and transform it in a short period into a larger project that opens new job opportunities (Masharaqa & Al-Silwadi, 2019). Paradoxically, despite recognizing the importance of developing students' creative abilities, not only in early education but also in higher education, there is an agreement that creativity and innovation have not received the necessary attention in university courses (Nassar, 2018).

Problem Statement & Research Questions: Higher education institutions are the first destination for graduates from schools, where they find themselves through studying a specialization, which honors their talents in it. Nevertheless, if we expect institutions to play this vital role in society and the economy, mechanisms are needed to explore the academics' perspectives in those institutions about creativity and innovation. They are the researchers, curriculum designers, and implementers of those curricula. Due to the vital role instructors play in developing students' creative potential, creativity and innovation should be a topic widely discussed in a higher education institution, to help them recognize and develop the future generation's creative and innovative abilities. This study will open the instructors' opportunity to start thinking of creativity and innovation and start establishing conditions for the flourishing of creativity in higher education classrooms.

Due to the lack of research that focuses on this topic in the Palestinian environment compared to other contexts in the global south, this study aims to identify the meaning of creativity and innovations from the viewpoint of faculty members at MOU University in the northern part of West Bank in Palestine. The study also seeks to be acquainted with the university curricula and its ingredients for developing creativity and innovation among university students. Moreover, understanding the kind of obstacles that prevent activating the curriculum role in promoting innovation and creativity among university students is another aim of the study Accordingly, to help achieve the presented objectives, the study answers the following main questions: - What do creativity and innovation mean from the academic's perspectives?- How can creativity and innovation be developed from the academic's views?- What are the main challenges that face academics from developing and fostering creativity and innovation?

The study highlights scientific importance by addressing a topic that is considered hot these days as we live in an era when the speed of innovations and inventions in science, culture, technology, and industrialization is more accelerated than any other time in humanity's history. The study highlights ways to develop university education to meet the local Palestinian market's needs and supply it with creative graduates who may solve Palestine's aggravated unemployment problem.On the practical level, this study's importance lies in providing decision-makers in universities scientific results and recommendations that may contribute to making the required change and development in the fields of knowledge creation, skills, and attitudes towards building creative and innovative graduates.

This study also identifies obstacles that prevent Palestinian students from achieving creativity and innovation to avoid and know the factors that enhance them. The present study brings practical ideas to the students said by university instructors themselves. It draws their attention to the labor market's primary skills or entrepreneurs and innovators in building their small projects. Moreover, the study will be an excellent guide for university academics by pinpointing the number of teaching strategies that can foster creativity and innovation skills.

Many believe that creativity and innovation are inherited characteristics that only some lucky people are born with it. Today, it is proved that these abilities universal among humankind and all at birth have this talent in varying degrees. (Fadaee & Alzahrh, 2014). Educationalists, researchers, and theorists have defined creativity differently; some look at it from the process dimension in which sees creativity is a mental process involved in a generation of new ideas (Hargreaves, 2007). Hence, the creativity process passes through several stages until it is complete. This process includes the mental maturity of the ideas and objectivity of judgments presented by the creative person.

For creativity to be based on the truth, it must be translated into a practical reality that others can benefit from as much as possible, whether based on evidence that proves it is true, realistic and correct.Others refer to creativity as the constellation of personality and intellectual traits shown by individuals who, when given a measure of freedom, spend significant amounts of time engaged in the creative process. (Edwards, McGoldrick, & Oliver, 2006). Al-Akhdar (2011) defined creativity as a mixture of abilities, preparations, and personal characteristics that can be enhanced by mental processes to lead to original and beneficial productions if any suitable environment exists. However. Most researchers and theorists adopt a creativity definition focused on the product, the outcome, and novelty (Edwards, McGoldrick, & Oliver, 2006).

In summary, although creativity and innovation are closely related, they differ since innovation depends on creativity to turn those creative ideas into use as products or as active practices. (Fadaee & Alzahrh, 2014). In other words, the notion of creativity includes ideas, inventions, and breakthroughs. On the other hand, innovation is the successful implementation of creative ideas within a specific context or environment (Louca, Varnava-Marouchou, Mihai, & Konis, 2014). From this point, innovators are creatives, but not all creative people are necessarily innovators (Fadaee & Alzahrh, 2014).

From the above definitions, we can see that creativity is composed of several factors, such as the cognitive abilities and processes involved in creative thinking and

the learning environment (Louca, Varnava-Marouchou, Mihai, & Konis, 2014; Fadaee & Alzahrh, 2014). Amabile (1998), Edwards, McGoldrick, & Oliver (2006) added intrinsic motivation because, Without intrinsic motivation, a creative thinker is unlikely to have the persistence required to solve a problem nor requiring knowledge and a willingness to challenge accepted wisdom.

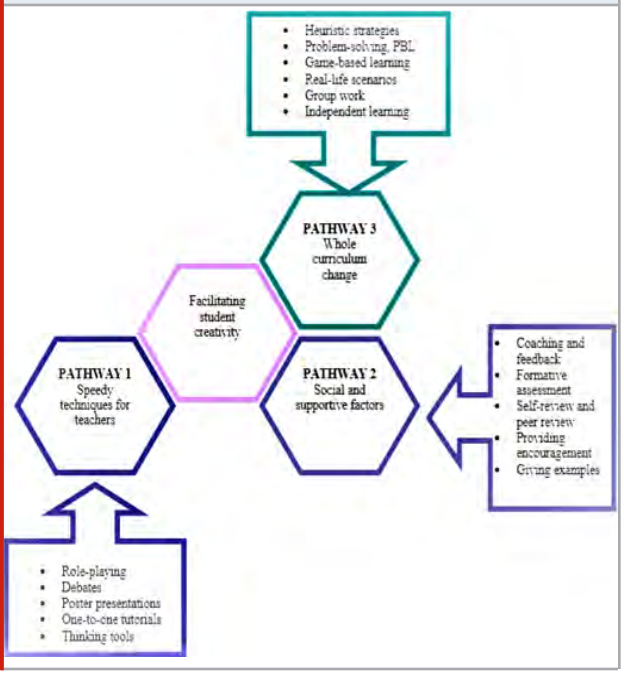
How to facilitate creativity and innovation in higher education?: Research conducted to nurture and facilitate creativity suggests that it can happen when individuals feel free from stress, safety, and positivity; This is important for the environment. Amabile (1998) did a comprehensive study that included three studies with three distinctly different subject populations. The interviewees were 120 scientists from over 20 various organizations, 16 marketing and development employees of the largest banks. For the third study, the interviews were 25 marketing and sales employees of significant railroads from the transcripts' content analysis.

Amabile (1998) revealed nine qualities of the environment that promote creativity; this fostering environment includes 1) freedom in deciding what to do and how to accomplish the task. 2) proper project management, which provides a good role model with excellent communication skills, protects the project team from outside distractions and interference. 3) sufficient resources with access to necessary resources, including facilities, equipment information, and people. 4) encouragement and being enthusiasm for the new ideas and creating an atmosphere free of threatening evaluation. 5) an environment where innovation is prized and failure is not fatal. 6) Recognition and showing a general sense that creative work will receive appropriate feedback. 7). It provides sufficient time to think creatively about the problem to explore different perspectives rather than impose an already determined approach. 8) a sense of challenge arising from the intriguing nature of the problem itself. 9).

The last quality of the environment that influences creativity and innovation is pressure and urgency, which is internally generated from the competition with an outside organization. Higher education graduates will be primarily creative when their task is enjoyable, stimulating, challenging, and when the group feels safe for its members (Roisio,2004). Donnelly & Barrett, (2008, p. 10) suggested detailed approaches for fostering student's creativity, and the approach includes three pathways. Pathway 1 encompasses several pedagogical strategies that can be integrated relatively quickly with students. All are creative, participatory teaching techniques that are essential tools in the teaching repertoire. These approaches involve high group member involvement while facilitating meaningful and fun learning through strategies such as role-playing, debates, poster presentations, one-to-one tutorials, and creative thinking techniques and tools.

Role-plays should be structured and have a defined set of participants with specific times, places, equipment, and rules. Debates are powerful teaching models because they help students' master concepts and learn to pursue goals effectively. Finally, with presentations, the student must understand what is being presented and whom it is being presented and apply appropriate presentation strategies.

Figure 1: Approaches for fostering students' creativity. Adopted from Donnelly & Barrett, (2008, p. 10)



Pathway 2 emphasizes the significance of feedback and support from an instructor who understands the creative process. More specifically, formative feedback (including self-review and peer review) can be used to prompt creative performance, develop artistic talent, and improve learning relationships. It can also can encourage students to reflect on their creative processes and working relationships, help students tap into powerful "creative flow" states, develop students' unique creative thinking and learning styles, help students explore strategies for more effective communication and collaboration.

Pathway 3 concerns fundamental curriculum change; the focus of education should be not on what students learn as to what they can do with their learning. He is mainly concerned with addressing cognitive development, mostly students' capacity to think and be happier. Any program that addresses creative education (Donnelly & Barrett, 2008) needs to cover every aspect of being creative, including motivational and emotional factors. The development of knowledge and skills, the capacity to imagine (primarily via the arts), and the capacity to solve fuzzy problems should be coved.

METHODOLOGY

Contextual background: This research was undertaken during the second semester of 2020 at MOU University. It is considered one of the largest Palestinian universities located in the Northern part of West Bank. It was founded in 1977. The university played a role in the Palestinian political and economic everyday life. Many prominent figures have emerged from it, who have made a significant contribution to the history of the Palestinian cause. The university has 11 faculties located and offers 103 undergraduate programmers, 23 diploma programs, and 66 graduate programs. The university is dedicated to promoting understanding, providing the highest quality undergraduate and graduate education, and serving as a leader in scientific research. The university acts as a base for sustainable development by encouraging students and the University community to assume leadership roles and participate in serving society. Study Participants: The sample was designed to include a range of disciplines and staff experience.

Table 1. Study Participants

Pseudonym Name	Gender	Major
Karim	Male	English Literature
Shakir	Male	Education
Huda	Female	Computer Engineering
Khaldoon	Male	Transport Engineering
Omar	Male	Economic
Noor	Male	Accounting
Durgham	Male	English literature
Saleh	Male	Pharmacy
Basheer	Male	Sports
Hamid	Male	Medicine
Nafez	Male	Sharia
Jameel	Male	Sharia
Suha	Female	Finance
Manal	Female	Physics
Mohamad	Male	Law
Gonaim	Male	Medicine
Rami	Male	Management
Jabi	Male	Law
Kefayah	Female	Education
Shareef	Male	Medicine

The participants were 20 academics at MOU University, holding PhDs and teaching graduate and undergraduate courses in several fields (Communication, Information Technology, Administration, Education, English Literature, Sharia, Medicine, Mathematics, Engineering, and Physical Education). Some professors are holding managerial and administrative positions in addition to their teaching responsibility, but their positions are not mentioned in the study in the protection of their privacy. Sixteen were male, and four were female. The table below summarizes the study participants.

Procedure and Analysis: Semi-structured interviews were carried out within the participants' office. The interviews were used to elicit perspectives, and each Interview lasted from 25-45 minutes. It was challenging to schedule a longer time to interview several professors due to their duties. A common semi-structured approach was adopted. The study objectives and goals were presented to all participants. Their verbal consent to participate in the study were taken before conducting the interviews. Participation in the survey was voluntary. The interview protocol was used as a guide, but the professors' responses determined course of the interviews. All the themes of interest were covered by most respondents, but not necessarily in the order listed in the protocol.

The interviews were recorded, transcribed, and coded in Arabic, relevant quotations were translated from Arabic to English and were determined according to study aim and questions. The confidentiality of the responses was assured to the participants, thus fulfilling the legal, ethical requirements. The translation was based on the meaning, as it is hard to have full meaning using verbatim translation. After the interviews were recorded and transcribed, they were analyzed by constant comparative categorization.

RESULTS AND DISCUSSION

This study aims to identify the meaning of creativity and innovations from the faculty members' viewpoints at MOU University and be acquainted with the university curricula and its ingredients for developing creativity and innovation among university students. Moreover, the study is also looking to understand the obstacles that challenge facilitating creativity and innovation in higher education institutions in Palestine. Accordingly, the study findings will be arranged in a way to help to achieve these goals. The findings section presents the perspectives of the academic of creativity and innovation concepts, and then highlights the facilitating factors of creativity and innovation in higher education. The findings will shed light on the importance of creativity and innovation in general and Palestinian context in specific. The section ends with a presentation to the main challenges that face creativity and innovation from the academic perspectives.

Creativity and Innovation Conception: According to the participants, "newness", "originality," "thinking outside the box," "benefit of self and community," and "problem-solving" are among the essential notions related to innovation and creativity.Innovation and creativity are "the student's ability to think in a way and is not necessarily identical to the teacher," Mr. Karim. It is "coming up with something new and find a team to develop and get out of the ordinary with something unfamiliar," Mr. Basheer. Ms. Huda believes that creativity and innovation refer to "teaching our

students a new way to solve problems facing us in our lives and focusing on students' critical thinking skills and not being limited to what the student takes or the teacher provides to students in the lecture."

Mr. Khaldoon believes that creativity and innovation are the new languages nowadays to overcome the unemployment challenge because "It is an opportunity to create employment and earning, but this opportunity will not be successful without serving the community." Therefore, students should think of unconventional ways to earn income other than employment.For Mr. Hamad, the creative person is: A person who can work successfully and becomes distinguished with limited resources. For example, a student from a low-income family with minimum resources and can succeed and become a successful engineer, doctor, or teacher; I can consider him creative and creative.

Mr. Saleh thinks what characterizes creativity is the "consistency in distinction" with "continuous development and improvement." On the other hand, innovation is transferring a good idea into tangible things and has a social, financial, or scientific impact." Creativity and innovation, according to Mr. Durgham, are different, and creativity comes before change. Creativity is "thinking outside the box and coming up with the new idea," while innovation is "converting those new ideas into the concrete project which has a financial return and benefits the community." Mr. Durham asked for an education system that encourages students to think outside the box.

Nature vs. Nurture: MOU University professors think that students can be creative with different degrees; they also believe that creativity and innovation cannot be taught or learned. It is the instructor and institution's responsibility to provide the fostering environment to spark creativity and innovation. "the teacher can urge students to be creative and guide their ideas and thinking but can't teach them creativity." Mr. Basheer elaborated by saying, "the most important thing is creating motivation and readiness among students, and this will not be through indoctrination."Due to the problematic situation Palestinians live under, Mr. Khaldoon thinks that instructors need to work hard, to build student's confidence "students do not have the confidence in themselves, and they can be creative." Doing that, according to him, is the first step to foster creativity. Mr. Saleh summarized this discussion by saying, "any human being and any society need creativity and innovation. %50 of creativity and innovation are based on innate genes, % 30 on society, environment and enthusiasm, and %20 percent on education".

Facilitating creativity and innovation in higher education: Academics at MOU highlighted the most recognized factor, which facilitates the nurturing of creativity in higher education. The institutional culture encourages teachers and instructors to think creatively and not bounded with instructions "It is important to give the teacher discretion, and there is no template for all teachers to use. The teacher knows his students," Mr. Durgham. Basheer also highlighted the need for changing the curricula and the evaluation system to encourage students to think outside the box "The educational process must be developed in all its aspects; our curricula and courses must be developed to avoid indoctrination and imitation. Evaluation methods also must be developed". The study participants discussed evaluation and assessment and they are covered in the challenges section.

No one can deny the critical role instructors play in the education process, and that was emphasized heavily among academics. According to Mr. Khaldoon, "A faculty member plays a major role in developing creativity and innovation. For example, when the teacher is positive and full of energy and vitality, she/he sends positive energy to students and makes them feel comfortable, and that encourages students to think openly and create". Mr. Hamad thinks that the teacher is responsible for developing creativity and innovation; the teacher "creates a successful or capable student, who can interact with the community and build its community." He continued saying, "the issue is not about a lesson that can be taught. It is a behavior in which the teacher behaves in front of his students and society". To help achieve that, Hamad elaborated more by saying, "the teacher must be a model of success so that students can emulate him/her and build their ideas from those around. The teacher must at least be convinced that students have creativity potentials". Mr. Shareef added to that by saying, "The teacher must believe in the idea of creativity and leadership to transfer it to students."

Ms. Huda & Mr. Hamad highlighted the role the university plays in fostering creativity and innovation and asserted on the importance of cooperation with the private sector to ensure sustainability "the university plays a significant role in supporting and developing leadership and creativity for the development to occur well, the cooperation of both the government, the private sector, and investors should be there." Ms. Huda.The importance of creativity and innovation: According to all participants, creativity and innovation are relevant and essential in higher education and graduate studies. Creativity and innovation are "gradual processes, and therefore it is impossible to develop it while students in their third or fourth year," Mr. Khaldoon. The study participants highlighted the point that university programs should be concerned about developing creativity and innovation, which should happen from the first academic year for the students.

According to Mr. Shakir, creativity and innovation are very important because they are the "requirements of the twenty-first century." These requirements are renewed and changed "if we looked at the required jobs in 2010, we find many of them fell in 2015, and expectations of jobs in 2030 will be different. Shakir. Thus, renewal is required". Ms. Huda elaborated on this issue by presenting the concept of the "skill gap," this gap refers to the difference between what students learn in the university

and requirement of the labor market. Hence, Ms. Huda thinks that creativity and innovation is a right solution for unemployment especially unemployment is very high in Palestine: "we should focus and work on initiatives and workshops to develop students' skills so they can come up or create a pioneering idea that employs these skills that have been developed for them."

One of the university goals, according to Mr. Hadi, is "to graduate students who will be active members of society after graduation, so if students will not be active members, it means that universities have not succeeded in achieving their goals." Accordingly, students in higher education must be prepared to think about the future step after graduation and "develop their personalities, creativity, and innovation skills" Mr. Noor.

Challenges that could face creativity and innovation at the University in Palestine: Despite the recognition of the importance of creativity and innovation for individuals and their achievement as well as for society, the development of creativity and innovation is still in its early steps. Professors, during their interviews, highlighted some challenges that face creativity and innovation in Palestine generally and in MOU University particularly.Although students are expected to be creative, creativity is seldom a clear objective of the learning assessment process. More specifically, looking at the types of assessments used in the Palestinian universities, including MOU, we see that the conventional paper exams with multiple choice and essay questions are preferred. According to Mr. Durgham, "there are policies at the university that limit the independence of the creative teacher, focusing too much on paper exams and having unified material and exams.

We must be freed from these restrictions to encourage creativity and innovation" Mr. Basheer agreed on the same point by stating, "the system used, regulations and laws limit creativity, such as the system used in the evaluation, and the lack of resources and awareness of the importance of creativity." The focus on exams and grades in the evaluation system creates a grade culture. According to Mr. Noor, "students are convinced that all learning depends on grades and that the question has the grade, grades became a community culture."Lack of "good mentorship and investment" is another challenge that faces creativity and innovation in higher education institution in Palestine according to Ms. Huda and this lack of mentorship is related to "the absence of institutions that support start-up opportunities, the absence of incubators for projects, so students start from them after graduation" Mr. Hamad. Other professors noted that excessive academic workload, not enough time to prepare lessons, large class sizes, and inadequate resources were factors considered that restrict the expression of creativity in higher education.

Recognizing the critical role faculties play in fostering creative thinking among their students, the primary objective of this study was to understand creativity and innovation conception from MOU university academic perspective. The findings revealed that the participants were aware of the importance of enhancing students' creativity in higher education, but creativity and innovation are not present in higher education classrooms. This agrees with other studies that explored creativity and innovation from academic perspectives (Alencar & Pereira, 2017; Edwards, McGoldrick, & Oliver, 2006 ; Masharaqa & Al-Silwadi, 2019). Despite this recognition, creativity and innovation in higher education are still in its early stages. MOU professors had a great time discussing how creativity and innovation are essential for the students themselves and their communities. At the same time, specific challenges are impeding their development in higher education. The next paragraphs will discuss these points in more detail and connect other literature.

Most pedagogical practice, as was indicated by the academics, is concentrated on the traditional way of teaching and focusing on accessing information "unfortunately, education in the university is still traditional" Ms. Huda. The traditional education means that it is teacher-driven education where students are gathered to learn and receive information based on teachers' knowledge. The students are unable to learn new things, and their experience is restricted to the education provided by the books and lecturers. Creativity and innovation, according to the participants, are related to "thinking outside the box," "benefit of self and community," "problem-solving," and "newness." To help realize that, students need to be agents and actors instead of recipients and audience. Creativity and innovation will not be acquired through indoctrination, will not be found in a specific course or a book. Students must be engaged in practical activities and connected to the community so they can think openly and creatively. Being connected with the community helps students also acquire problem-solving, critical thinking, and research skills.

The education system at MOU University is not different from any other education system, according to (Louca et al., 2014). Accordingly, the educational system in many countries does not promote creative teaching/learning processes (Ferrari et al., 2009; Robinson, 2006).

The participants' thoughts of fostering creativity and innovation are significantly connected to pathway 1 in Donnelly & Barrett, (2008) model, as presented in the earlier section. Pathway 1 encompasses several pedagogical strategies that teachers can employ in the classroom to encourage students' participation and envelopment. Students have the potential for creative living, which is a combination of nature and nurture, as was discussed by the participants. This suggests that some students have some upper limit of their creative capabilities, but they need to be guided toward achieving their goals. Creativity and innovation are not taught in a specific course as pointed by all participants but are acquired gradually and indirectly. Donnelly & Barrett, (2008) model emphasized in pathway three that the

education should be not so much on what students learn as on what they can do with their learning.

Teachers can guide students on the basic principles of creativity and innovation and encourage them to apply them in real cases or situations. Teachers can guide students' thoughts and ideas; they can drive students' interest and motivation but cannot teach them the knowledge of creativity and innovation and ask them to memorize it. The research findings show a strong relationship between teaching for creativity and teaching creatively, being a role model for students reflecting innovative ideas, experience, and be a great way to teach them creativity. Teachers' ability to transfer his/her course to be more practical, teachers' behavior and great personality driver to creativity and innovation because students are more likely to accept the notion of creativity and innovation from creative teachers.

CONCLUSION

Today, it is crucial to be innovative more than ever, manages a changing environment, respond to technological advances, evolving customer needs, and globalization. Educational practice requires not only keeping pace but also being proactive in meeting the needs of the workforce. This research contributes to a discussion of innovation and creativity and the university's role in creating productive and successful graduates with critical and necessary skills. Besides, it explains more about Masharaqa & Al-Silwadi, (2019) findings regardings the lack of creativity, leadership charactersitcs among Palestianin graduates. This paper argues for the need to mix innovation theory with practice and create experimental and participatory courses to ensure students preparedness for real-life challenges. This research is presented to start this discussion. It seems undeniable that creativity and innovation are essential to organizations, and the role of the university is to prepare students for the demands of industry and the labor market. Consequently, the study recommends building partner relationship between universities and the private sector taking into account the nature of the work of the sector and graduate specialties.

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Efficacy of Methanolic Extract of Fruit Pulp and Leaf of *Terminalia chebula* and *Aegles marmelos* Against *Staphylococcus aureus*

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ABSTRACT

Plants owe eminence in healing and curing dreadful diseases from vedic civilization. The present investigation includes *Aegles marmelo*s (Baelpatra) and *Terminalia chebula* (Harad) as potential plant entities to be used against *Staphylocoocus aureus*. Methicillin Resistant *S. aureus* (MRSA) is major cause of nosocomial infections and are very difficult to cure because these strains are resistant to almost all clinically available antibiotics. Although present research did not employ MRSA strain being pathogenic but activity against susceptible strains offers propensity of these sacred plants to be exploited for bacterial conjunctivitis cure in India. The fruit pulp excluding seed and outer covering of *Terminalia chebula* and *Aegle marmelos* were used as plant part to prepare methanolic extracts using standard methods. The methanolic extracts were diluted two fold starting from a higher concentration of 250mg/ml to 0.97mg/ml and were tested against *Staphylococcus aureus* and *P. aeruginosa* using agar well diffusion assay. In addition methanolic extract of leaf as plant part from both plants were also tested starting from a higher concentration of 10mg/ml to 1.25mg/ml using two fold dilution. DMSO was used as control.Antimicrobial analysis confirmed that the plant contains bioactive compounds that exhibit measurable antimicrobial activity against selected bacteria.The zone size of 24mm using Baelpatra and 26mm using *Terminalia* at a higher concentration of 250mg/ml reveal the significance of present work to be exploited against resistant strains of *staphylococcus*. Although the plants possess a number of pharmacological activities due to the presence of bioactive compounds, very little work has been done on this potential medicinal applications of fruit extracts of plant against the diseases particularly on multidrug resistant bacterial pathogens. Researchers need to exploit these medicinal plants as good candidates to overcome developing resistant of antibiotics to infectious disease which are caused by these microorganisms.

KEY WORDS: *T. CHEBULA*, ANTIMICROBIAL, ZONE OF INHIBITION, CONJUNCTIVITIS, MRSA, *AEGLE MARMELOS*.

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INTRODUCTION

Aegle marmelos belongs to family *Rutaceae* and is one of the most important medicinal plants since times of Sage Charaka (1500B.C.). It is a native plant of India commonly called "Bael" and "Temple Garden Plant". The leaves are used to worship Lord Shiva. The plant is a slow growing 25-30 feet tall tree. Flowers are greenish white in color with peculiar fragrance. The tree is subtropical plant growing well in dry forests, hilly

and plain areas. It is found in India Ceylon, Sri Lanka, Pakistan, Malaysia, Nepal, Cambodia, Thailand and in almost all states of India. The plant has been attributed to have enormous ethno-medicinal applications in medical health care. Vedic literature has reported the plant to treat jaundice, constipation, bronchitis, snake-bite, abdominal discomfort, Spermatorrhoea, Leucoderma, eye disorders, and ulcers.

Its Fruit is a rich source of nutrition as the pulp consists of water, sugar, protein, fibers, fat, calcium, phosphorus, potassium, iron minerals, and vitamins A, B, and C. The leaves cause infertility or abortive action in women. The plant possesses antioxidant activity and aids in fighting against gastrointestinal and cardiovascular disorders. The plant has been cited to be antidiabetic, antimicrobial, and anti-inflammatory in literature (Sekar et al., 2011). *Aegle marmelos* is known to be enriched bioreserve of more than hundred phytoconstituents extracted from different plant parts namely terpenoids, steroids, phenols, *flavonoid alkaloids*, cardiac glycosides and saponins which in turn have been reported to be well known biological agents against chronic disorders and as boosters of immunity(Devi et al., 2020). The plant is also reported to have strong antioxidant potential as it effectively scavenges the free radicals(Chaubey et al., 2020).

The leaves have been considered as a rich repository of phytochemicals in comparison to other conventional fruits and shows promising results for curing eye, ear, and skin infections. The dried fruit pulp has anti diarrheal activity and shows activity against several pathogens associated with dysentery. The aqueous extract of unripe fruit is recommended as a potent agent in fighting against Rota virus and Giardia. Methanolic extracts from leaves have been reported to have antidiabetic activity. *Terminalia chebula* commonly known as "Harad" belongs to family *Combretaceae* found in the forests of Northern India, Uttar Pradesh, Bengal and is very common in existence in Southern part of India.

The plant is a medium to large sized tree distributed throughout tropical and subtropical Asia including China (Rao et al., 2011). The plant is widely consumed in China and is referred as "Tibet Olive", the fruit peel revealed the presence of 29 compounds and was confirmed through Ultra High Performance Liquid Chromatography(Li et al, 2019). *T.chebula* has anti-inflammatory, neuroprotective, hepatoprotective, cytotoxic and antidiabetic properties(Nigam et al., 2020). In another study, the plant was found to regulate the lipid profile and reduce inflammation in Diabetic patients (Pingali et al. 2020).

Tribal people in Tamil Nadu, Karnataka routinely use Harad to cure several ailments like fever, cough, diarrhea, gastroenteritis, skin disease, urinary tract infection (Dash, 1991). The antimicrobial activity of this plant has been reported against several bacterial strains using fruit pulp (Dutta et al., 1998; Malckzadeh et al, 2001). The plant part (fruit pulp) has been tested against *H. pylori, X. campestris*, and *S. typhi*. The plant fruit pulp is also reported to be effective against a number of dermatophytes and yeasts. The fruit of the plant possess complex antimicrobial compounds to cure diseases like digestive and cardiovascular ailments along with pathogenic bacteria (Bag et al., 2009).

Our present research compared the potential of fruit pulp to evaluate antimicrobial activity against bacteria. Bacterial conjunctivitis is less epidemic with 135 in 10,000 cases of incidence reported. It can be contacted directly from infected individuals or due to proliferation of native conjunctival flora. Contaminated fingers and occulo-genital spread are most common ways of spreading bacterial infections. The most common pathogen for bacterial conjunctivitis is Staphylococcal species followed by *Streptococcus pneumoniae* and *H. influenzae*. Children are more susceptible to *H. influenzae, S. pneumoniae* and *Moraxella catarrhalis* (Ronnerstain et al., 1985; Bag et al., 2009). The plant has Gallic acid which was found to prevent the growth of esophageal cancer in a study(Sun et al., 2020).

Conjunctiva is a thin translucent membrane, lining the anterior part of sclera and inside of the eyelids which in turn has two parts-Bulbar and Palpebral. Bulbar part starts at the edge of the cornea and covers the visible part of sclera; the palpebral part lines the inside of the eyelids. Infection of conjunctiva is known as conjunctivitis and characterized by dilation of the conjunctival vessels which in turn results in edema of the conjunctiva typically associated with discharge. Majority of bacterial conjunctivitis cases (50-75%) have been reported in children. It is more frequent from December to April (Horven, 1993; Morrow and Abbott, 1998; Epling and Smucny, 2005; Hovding, 2008).

Conjunctivitis can be infectious and non-infectious. Infectious type is owed to virus and bacteria whereas non-infectious is allergic, toxic and cicatricial. Further classification includes acute, hyperacute and chronic depending upon mode of onset and severity. The red eye disease should be differentially diagnosed from other ocular disease which has similar symptoms. Purulent and muco-purulent discharge is due to bacterial infection while watery discharge is due to viral conjunctivitis. In comparison, itching is due to allergic conjunctivitis. The course of infection ends in 7 to 10 days and if the problem persists, one should refer to ophthalmologist (Smith et al., 2009). A 2005 study showed that the economic impact of bacterial conjunctivitis is significant and ranges between $377 million to $857 million in America (Ta et al., 2020).

Decreased vision and purulent discharge are generally observed in hyperacute bacterial conjunctivitis accompanied with eyelid swelling, eye-pain, palpitation, and preauricular adenopathy. Chronic conjunctivitis is referred to prolonged infection of 4 weeks with *S. aureus, M. lacunata* and enteric bacteria being most common cause (Yannof and Duker, 2004). At least 60% cases of suspected acute bacterial conjunctivitis are self-limiting

within 1-2 weeks of initialization. Topical antibiotics seem to be more effective in patients who have acute bacterial infection. All broad-spectrum antibiotics eye drops seem in general to be effective in treating bacterial conjunctivitis. Alternate to antibiotic therapy fortified Vancomycin or ophthalmologist is only option for suspected cases (Shanmugnathan, 2005; Freidlin et al., 2007). A recent study on bacterial conjunctivitis patients confirmed that 17% of the patients had polybacterial infection and 83% had monobacterial infection which showed that there is a need for broad spectrum Opthalmic drugs (DeCory et al., 2020).

MATERIAL AND METHODS

Plant and culture collection: The fruits of *T. chebula* and *A. marmelos* are used in the present study for antimicrobial activity and were procured from Kurukshetra university campus itself and identified from Botany Department of Kurukshetra University, Kurukshetra, Haryana, India. The human pathogenic microorganisms were procured from Microbial Type Culture Collection (MTCC) Institute of Microbial Technology (IMTECH), Chandigarh; which included Gram-negative bacteria: *P. aeruginosa* (MTCC-2295) and Gram-positive bacteria: *S. aureus* (MTCC -3160).

Preparation of *Terminalia chebula* fruit pulp extract: The fruit pulp was extracted by removing seed and outer covering of *Terminalia chebula* and were thoroughly washed with water, allowed for oven drying at 50-60° C for 3-4 hours and grounded into fine powder. The 20 gm of this powder was soaked in 100 ml of methanol, and incubated for 72 hr. at room temperature. The extracts were filtered with Whatman filter paper. The extra solvent from the filtrate were evaporated by using water bath at 45-50 °C. The residual powder after solvent extraction was dissolved in DMSO and stored at 4° C.

Preparation of *Aegle marmelos* fruit pulp extract: The fruits of *Aegle marmelos* were thoroughly washed with water then allowed for oven drying at 50-60 °C for 3-4 hours and grounded into fine powder. The 20 gm of this powder was soaked in 100 ml of methanol and incubated for 72 hours at room temperature. The extracts were filtered with Whatman filter paper. The extra solvent from the filtrate was evaporated by using water bath at 45-50° C. The residual powder after solvent extraction was dissolved in DMSO and stored at 4° C.(Bag et al., 2009)(Ganpathy et al., 2016).

Antimicrobial activity of plant extracts by Agar Well Diffusion Assay: The antimicrobial activities of plant extracts were evaluated by agar well diffusion assay (Pereze et al., 1990). The microbial inoculums were inoculated aseptically and spread uniformly on surface of pre solidified Mueller Hinton Agar (MHA) plates with the help of sterile glass spreader or sterile cotton swabs. A well of about 6.0 mm diameter was aseptically punctured using a sterile cork borer. The cut agar was carefully removed by the use of sterile forceps. DMSO was used as a negative control. The Petri Plates were kept in laminar for 30 minutes for pre-diffusion to occur and then Petri Plates were incubated overnight at 37 °C for 24 hours. The antimicrobial spectrum of extract was determined in terms of zone sizes (inhibition zone diameters) around each well. Zones were measured by high media zone scale.

RESULTS AND DISCUSSION

The increasing rate of mortality among developing countries can be assigned to infectious diseases which are aggravated due to rapid resistance developed against pathogenic bacteria including most pathogenic *S. aureus*. The incidence of *S. aureus* disease infection and complications has increased abruptly in recent years because of increased frequency of invasive procedures which led to resistance of *S. aureus* strains to the available antibiotics. Various researchers have reported the efficacy of several medicinal plants against *S. aureus*. The antibacterial activity of ten medicinal plant extracts on antibiotic resistant bacteria which includes *P. granatum* (pomegranate), *A. millifolium* (yarrow), *C. aromaticus* (clove), *M. officinalis* (lemonbalm), *O. basilucum* (basil), *P. guajava* (guava), *R. officinalis* (rosemary) and *S. officinalis* (Sage) alongwith *T. vulgaris* (Thyme) and *Syzygium joabolanum* (Jambolan).Highest activity was observed using plant extracts of *C. aromaticus* (clove) while no activity was resulted in *A. millifolium* and *S. officinalis*. *Syzygium* resulted in MIC against *S. aureus* at 300 ppm (Gislene et al., 2016).

Chandra et al. (2013) evaluated antimicrobial activity of medicinal plants against human pathogenic bacteria. Leaf extract of two medicinal plants *Lagerstroemia indica* and *Annona reticulata* was extracted using aqueous and methanolic solvent systems against *K. pneumoniae, S. aureus, S. typhi, P. vulgaris* and *P. aeruginosa*. Methanolic extracts were found to be better solvent system than aqueous extracts. *L. indica* resulted in a zone of 12mm and 8mm while *A. reticulata* resulted in zones of 11mm and 10mm using methanol and aqueous extracts respectively. Mohammed et al. (2018) carried out agar well diffusion assay to evaluate antimicrobial activity and used refluxed methanolic extracts and macerated methanolic extracts of *B. vulgaris, C. augustifolia, C. cassia, C. monspeliensis, N. sativa,P. granatum, R. tripartata, W. frutescens* and *Zingiber officinalis* against gram positive and gram negative strains.

The zones of inhibition ranged from 6.0 to 23.0mm while MIC ranged from 0.1 to 12.8 mg/ml. *Berberis vulgaris*, *Cistus monspeliensis* and *P. granatum* resulted in highest activity against S. aureus resulting in zones of 12.0 and 23.0,17.0 and 16.0, 20.0 and 20.0 mm in refluxed methanolic extract and macerated methanolic extracts respectively. In another study, water and organic solvent extracts (methanol, ethanol, petroleum ether and chloroform) of five medicinal plants against seven bacetrial pathogens. *A. calamus, T. bellerica, N. arbortritis, C. borivilianum* and *E. cardamomum* leaf and fruit extracts were tested against *E. coli, S. aureus, P. aeruginosa, S. typhii, S. pyogenes, P. mirabilis* and *A.*

baumani (Khatri et al., 2016; Mohammed et al., 2018).

The methanolic extracts of *T. bellerica* resulted in a zone of 22mm which was highest while C. *borivilianum* resulted in a zone of 21mm against both *S. typhi* and *S. aureus* using chloroform and ethanolic extracts respectively. Ethanolic extracts of selected medicinal plants were tested against *S. aureus, S.marcescens, S. saprophyticus, S. pneumoniae, S. pyogenes, A. baumannii, E. faecalis* and *P. mirabilis* using broth microdilution method. Maximum inhibition concentration was showed by ethanolic extracts of *Sasamum indicum* of 100 ppm against *S. aureus, S. pneumoniae, S. pyogenes, A. baumannii, E. faecalis* and *P. mirabilis*. MIC values ranged from 25-100ppm (Shahla et al., 2014). *Rachuonyo* and his coworkers (2016) reported antibacetrial activity of methanolic leaf extracts of *A. secundiflora, B. frutescens, T. minuta* and *V. lasiopus* against *S. aureus*. *Tagetes minuta* was found to be most active even at low concentration with a MIC value of 8.9 mg/ml and MBC value of 10 mg/mL while *Vernoia lasiopus* resulted in a MIC value of 12.2mg/ml and MBC value of 14.2mg/ml. They also reported the presence of flavonoids, alkaloids,tannins and saponins in all extracts (Rachuonyo et al. 2016).

Bishnu and his coassociates (2015) evaluated antimicrobial activity of 16 traditionally available medicinal plants of Nepal against 13 clinical, 2 reference bacterial species using Microbroth Dilution Method. The research reported that *Cynodon dactylon* ethanolic extracts resulted in moderate activity against MRSA and 13 bacterial strains while chloroform extracts found to be best against *S.aureus* giving a MIC value of 31µg/ml. Usmaan Ali Khan et al. (2013) reported antibacterial activity of *Bergenia ciliata, Jasminium officinale*, *Santalum album* using agar well diffusion assay against *B. subtilis, P.vulgaris, E. coli, P. aeruginosa* and *S. aureus* by using hot and cold aqueous extracts. The roots of *B. ciliata* cold water extracts showed highest activity against pathogens resulting in a zone of 16mm against *S. aureus* while highest 19mm against *B. subtilis* (Bishnu et al. 2015). In another study, the macerated methanolic plant extracts of *Dacryodes edulis* showed significant activity against all *S.aureus* isolates with MIC value 64-256 ug/ml.

Ocimum gratissimum showed inhibitory activity on 9 out of 11 isolates of *S. aureus* while *Commelina erecta* and *Spilanthes filicaulis* revealed similar results on 6 out of of 11 clinical isolates. The study also elucidated the correlation between phytochemicals present in these plants which are the key route to significant antimicrobial activity. The presence of saponins alongwith alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, tannins and triterpenes were correlated to inhibit the *S. aureus* strain even at low concentrations (Leonard Sama Fonkeng et al., 2015). In another study, the ethanolic extracts of *Punica granatum, Syzygium aromaticum, Zingiber officinalis* and *Thymus vulgaris* were found to be effective against *S. aureus*. At a concentration of 10mg/ml *Cuminum cyminum* was effective against *S. aureus* only while other plants show inhibitory activity against both *S. aureus* and *P. aeruginosa* with a MIC value of 2.5-5.0mg/ml and MBC value of 5.0 and 10mg/ml (Leonard Sama Fonkeng et al., 2015). In another study, the *L. camara* based Silver nanoparticle solution was found to be highly effective against *S.aureus* (Aritonang et al.,2020).

Table 1. Inhibition zone diameters (in mm) of methanolic fruit pulp extract of *T. chebula*.

Conc. (mg/ml)	250	150	125	62.5	31.25	15.625	7.81	3.90	1.95	0.97	DMSO
Zone of Inhibition against *S. aureus*	26	24	24	21	19	16	12	-	-	-	-
Zone of Inhibition against *P. aeruginosa*	20	19	18	17	17	15	13	-	-	-	-

Table 2. Inhibition zone diameters (in mm) of methanolic fruit pulp extract of *A. marmelos*.

Conc. (mg/ml)	250	150	125	62.5	31.25	15.625	7.81	3.90	1.95	0.97	DMSO
S. aureus	24	24	21	18	14	-	-	-	-	-	-
P. aeruginosa	14	12	12	10	8	6		-	-	-	-

Terminalia chebula–Harad and Aegle marmelos**–Baelpatra propensity against** *S. aureus***:** Methanolic extracts were prepared using fruit pulp were found to possess significant antimicrobial activity against Gram Positive *S. aureus* and Gram Negative *P. aeruginosa* bacteria compared to standard taken as DMSO. The extracts of fruit pulp have been assessed for antimicrobial activity against *P. aeruginosa* and *S. aureus* at ten different concentrations (250 mg/ml to 0.97 mg/ml) as shown in Table 1. Starting from a higher concentration of 250 mg/ml two fold dilutions were made using DMSO resulting in concentration of 150, 125, 62.5, 31.25, 15.625 mg/

ml and so on till a final concentration of 0.97 mg/ml (Table 1, 2 and 3).

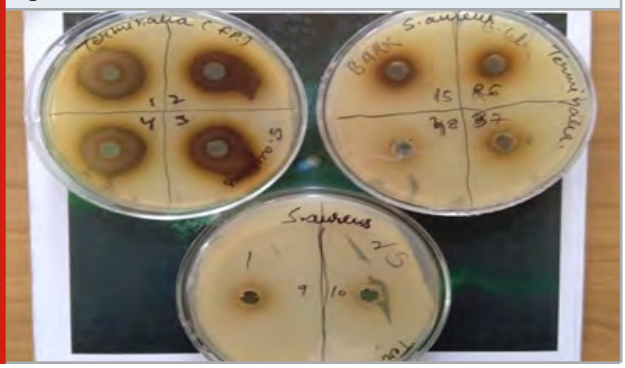
The volume of all different dilutions was kept same as 100 µl and media plates were incubated and afterwards the standard procedure of agar well diffusion assay was carried out. The agar well diffusion method was used to evaluate the antimicrobial activity by measuring the inhibition zones against the test microorganism. A zone of 26 mm was observed at higher concentration of 250 mg/ml. The zone size reduced to 24 mm on two fold dilutions of 250 mg/ml and remains constant at two concentrations of 150 and 125 mg/ml (given in Fig-1 and Fig-2).

Table 3. Inhibition zone diameters (in mm) of methanolic extracts of leaf of *T. chebula* and *A. marmelos* against *S. aureus* and *P. aeruginosa*.

Sr. no.	Concentration of extracts(mg/ml)	Leaf(T) *S. aureus*	Leaf(T) *P. aeruginosa*	Leaf(A) *S. aureus*	Leaf(A) *P. aeruginosa*
1.	10	17	19	14	10
2.	5	16	17	10	6
3.	2.5	14	15	7	6
4.	1.25	13	15	7	-

On further dilution the zone size of 21 mm was observed at 62.5 mg/ml which showed positive correlation with decrease in concentration. A zone of 19 mm at 31.25 mg/ml and zone of 16 mm at 15.625 mg/ml further validated that increasing plant extracts have positive effects on zone size or say inhibition rate against *S. aureus*. While a zone of 12 mm was reported to be final zone at 7.81 mg/ml because on further dilutions no zone of inhibition was observed. It can be assumed that lower concentrations were unable to inhibit *S. aureus*. But results obtained showed potential approach to exploit fruit pulp of Harad plant to be used against different strains of *S. aureus* to explicit its resistance developed so far. Ghosh et al. (2011) .

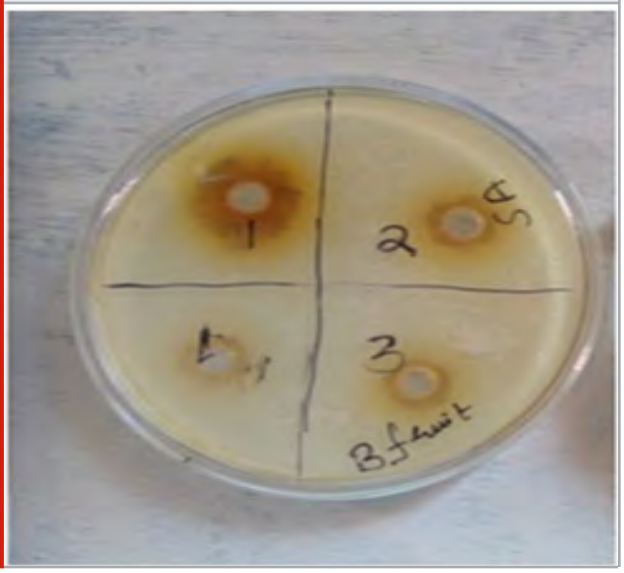
Figure 1: Terminalia fruit pulp showing zones of inhibition against *S. aureus*.



Reported similar findings in methanolic leaf extracts of *Terminalia chebula* exhibited higher antimicrobial potential amongst all plant parts used followed by fruit extracts showing effective antimicrobial activity in five different medicinal plants viz; *T. bellerica, T. chebula, E. officinalis, Punica granatum* and *Lawsonia inermis*. Samy and Ignachimuthu, 2000 reported similar results in *C.auriculata* exhibiting significant antimicrobial activity against *E. coli* and *S. aureus* at a concentration of 6mg/ml which correlate to present investigation where a minimum zone of inhibition of 12mm was observed at 7.81 mg/ml (Samy and Ignachimuthu, 2000).In a recent study the dried leaf extract of *T.chebula* seeds gave a zone of 13mm against *S. aureus* at a concentration of 250µg/ml(Krishna et al., 2020).

The results reported were in corroboration to the earlier reults where methanolic extracts have been found to be more effective as compared to other solvents (Ghosh et al., 2011). Fruit pulp extract showed a minimum inhibition zone of 13mm in methanolic extracts when taken as MIC assay. While on testing against *P. aeruginosa* agar well diffusion assay resulted in a zone of 20mm at 250mg/ml which showed constant decrease in zone size with further dilution to 0.97mg/ml. A zone of 19mm at (150), 18mm at (125),17mm at (62.5 and 31.25), 15mm at (15.625) and a zone of 13mm at 7.81mg/ml were observed and were in well accordance to decreasing concentration or dilutions.

Figure 2: Baelpatra fruit pulp showing zones of inhibition against *S. aureus*



Similar to previous zones against *S. aureus* no further zones were resulted at lower concentrations than 7.81mg/ml. Kannan et al. (2009) reported the potential of using dry fruit extracts even at 1 mg/ml to possess broad spectrum activity showing the ethanolic extracts of *Terminalia chebula* using fruit extracts to be effective against *S. typhi ,S. epidermidis, S. aureus, B. subtilis* and *P. aeruginosa*. In comparison to fruit pulp the leaves of the plants were also exploited to test efficacy in inhibiting both *S. aureus* and *P. aeruginosa*. The leaves were washed dried and methanolic extracts were prepared using standard preparation methods.

However, the investigation researched the activity at lower concentration than that of fruit pulp. As in both cases the fruit pulp was limited to generate zone of inhibition approximately near 10 mg/ml so the lower concentrations were prepared starting from 10 mg/ml and serially diluted two fold resulting in concentration of 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml (Table-2). A zone of 17 mm was observed at 10 mg/ml using methanolic leaf extract of *Terminalia* followed by zone of 16 mm at 5 mg/ml, 14 mm at 2.5 and a minimum size zone of 11 mm at 1.25 mg/ml. So, the leaf as plant part is more effective than fruit pulp against *S. aureus* (Kannan et al., 2009).

Similar findings were observed in Baelpatra where the zones were although of smaller size but can be considered resulting in zone of 14 mm at 10 mg/ml followed by 10 mm at 5 mg/ml, 7 mm at 2.5 mg/ml and a similar zone of 7 mm at 1.25 mg/ml against *S. aureus*. While inhibition zones against *P. aeruginosa* were more prominent using *Terminalia* leaf extracts. A zone of 19 mm was observed at 10 mg/ml followed by 17 mm at 5 mg/ml ,15 mm at 2.5 mg/ml and 15 mm at least concentration of 1.25 mg/ml. The results depict better efficacy of leaf extracts against P. aeruginosa than fruit pulp.

The fruit pulp of Terminalia is more effective against *S. aureus*. In comparison Baelpatra resulted in zones of 10 mm at 10 mg/ml followed by a zone of 6 mm at 5 and 2.5 mg/ml while no zone was observed at 1.25 mg/ml. Although traditional wisdom indicates the importance of using these trees in historic times and reveal their potential in using against *S. aureus* a multi strain bacterium which developed resistance in present scenario. Similar agar well diffusion assay was performed for *A. marmelos* fruit pulp extract starting from 250 mg/ml as initial concentration using methanol against *S. aureus*. Two fold dilutions were made using DMSO and same is used as control. A similar zone of 24 mm was observed at 250 mg/ml followed by 24 mm at 150 mg/ml, 21 mm at 125 mg/ml.

The zone size decreased to 18 mm at 62.5 mg/ml and further goes on decreasing to 14 mm at 31.25 mg/ml. As compared to Terminalia (Harad) which resulted in inhibition zones at further two concentrations of 15.625 and 7.81 mg/ml Baelpatra fruit pulp extract showed no zone of inhibition on these concentrations while as compared to *S. aureus* agar well diffusion assay performed against *P. aeruginosa* resulted in inhibition zones at initial five concentrations. A zone of 14 mm was observed at 250 mg/ml which in turn is followed by a zone of 12 mm at 150 mg/ml and 125 mg/ml, 10 mm at 62.5 mg/ml followed by 8 mm at 31.25 mg/ml. The least size zone of 6 mm at 15.625 mg/ml Table-2.

The zone size obtained were in corroboration of Poonkothai and Saravanan (2008) who reported antimicrobial activity of different plant parts of Baelpatra such as leaf, bark and fruit extracts using methanolic, chloroform and aqueous extracts using disc diffusion assay against seven pathogens which includes S.aureus, *B. subtilis, P. mirabilis, E. coli, K. pneumoniae, S. paratyphi A, Salmonella paratyphi B. Methanolic* extract reported to be more effective in inhibiting the pathogens tested in comparison to chloroform which was more effective solvent than aqueous extracts, but the zone of inhibition observed were less effective as compared to commercial antibiotic while discussing in concern to MRSA the zone of inhibition obtained using methanol as solvent resulted in zone size of 14 mm (leaf), 7 mm (bark), 11 mm (fruits)while chloroform extracts resulted in zones of smaller sizes like 6 mm, 2 mm and 5 mm using leaf, bark and fruit respectively. The aqueous extracts produced a zone of 2 mm using leaf while a zone of 10 mm using fruits as plant part while bark did not result in any zone of inhibition. The differential zones of inhibition observed were attributed to differential polarity and non-polarity of constituents extracted using different solvents (Suresh et al., 2009).

Leaves and flowers of *Aegle marmelos* were extracted using methanol as solvent system at three different concentrations 50, 100, 200 ppm against *E. coli, P. aeruginosa, P. mirabilis, S. typhi, S. aureus* using disc diffusion method. *E. coli* was found to be more susceptible due to tannin alkaloids giving a significant zones against *E. coli* (17 mm), *S.typhi* (17 mm), *S.aureus* (15 mm), *P. aeruginosa* (13 mm) at all concentration but the highest concentration of 200 ppm did not revealed marked differences using leaves as plant parts. Flowers extract prepared using methanol as solvent system resulted in highest activity against *S.aureus* giving a zone of 18 mm at 200 ppm similar to *P. mirabilis* (18 mm), 16 mm against *P. aeruginosa* and 15 mm against *E. coli* and *S.typhi* and the flavonoids were found to be main constituents in flowers responsible for this activity.

Sridhar et al., 2014 reported antibacterial and anti-helmintic and cardiotonic activities using aqueous and ethanolic extracts of dried fruits of *Aegle marmelos* using cup diffusion method. Ethanolic extracts resulted in zones of 18 mm while aqueous extract gave a zone of 19 mm against *E. coli*. MIC assay revealed a zone of 6.25 µg/ml against *E. coli*. Karumaran et al., (2016) observed highest zone of inhibition of 20mm using acetone and hexane extracts at 10 mg /ml concentration against *P. aeruginosa* and *B. subtilis*. Lowest zone of 5 mm was observed against *K. pneumoniae* at same concentration in acetone extracts (Sridhar et al., 2014). In another study,

the Minimum Inhibitory Concentration of *A. marmelos* against *S. aureus* was found to be 31.25µg/ml (Owk et al., 2020). Acetone extracts showed a zone of 16 mm against *S. aureus*, 20 mm against *P. aeruginosa* and *B. subtilis,* 11 mm against *E. coli* and gave a MIC value of 10.5 mg/ml for both acetone and ethanol extracts. Rajan et al., (2011) reported that fruit pulp is used as a remedy for gastrointestinal infections of human.

Antioxidant potential of fruit pulp extract showed the presence of steroids, terpenoids, saponins, tannins, lignin, and flavonoids. The plant is a perennial tree found wild in sub Himalayan tract, central and south India. Fruits have greatest medicinal values. Mujeeb et al., (2014) screened the phytoconstituents using aqueous and methanolic leaf extracts which revealed the presence of alkaloids, flavonoids and phenols observed highest inhibitory activity of aqueous extracts against *S.epidermidis*, while methanolic extracts showed more potent activity against *S.aureus* at 40mg/ml. IC values revealed presence of aldehydes, flavonoids, fatty acids, methyl esters, terpenoids, phenolics, steroids and aromatic compounds along with alcohols (Mujeeb et al., 2014). A recent Myanmar based study found that the plant extract of *A.marmelos* was found to be toxic against HeLa Cell Line and hence the plant constituents can be related with anti-cancerous properties (Aung et al., 2020).

CONCLUSION

Phytochemicals present in these plants are key route to significant antimicrobial activity. The presence of saponins alongwith alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, tannins and triterpenes were correlated to inhibit the *S. aureus* strain even at low concentrations. The *T. chebula* extract showed inhibition zones at 7.81 mg/ml whereas *A. marmelos* extract didn't show significant zones after 31.25mg/ml which shows that *T. chebula* is densely packed with phyto-constituents and can be referred to be more potent for curing Conjunctivitis.The bioactive substances from these plants can be employed in the formulation of antimicrobial agents for the treatment of various bacterial infections. The results of present investigation indicate that antibacterial activity varies with the plant part and solvent extract concentration. Further, the research needs to be done for finding potential solutions for Multi Drug Resistant pathogens.

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Spinal Column Diagnosis in Cervical Vertebrae Interface Using Image Spectral Techniques

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ABSTRACT

With the time, as there is advancement in medical technology, there is also a huge increment in number of physical disabilities and diseases. Among them some are curable and some aren't. Low back pain & sub-acute pain growing simultaneously. Ignorance of such pain becomes chronic disease which not only affect our spine, but also affect our work. Cervical traction is most preferred treatment for cervical spine disabilities in intervertebral disc, the vertebrae, the ligaments, and the facet joints but its effectiveness as a part of physical therapy is a matter of discussion. First step for spinal column diagnosis is vertebra detection. Due to presence of skull bone in C-spine region this task becomes more difficult as X-ray images considered are noisy low contrast images. In this paper an automatic approach is proposed for calculating intervertebral posterior height and based on the distance between different vertebrae traction force at C-spine region is calculated. The Proposed method consists of following steps: 1) Vertebra detection 2) Intervertebral posterior height calculation 3) calculating traction weight. Data of 17 volunteers and X-ray Image data of 12 cases are taken to validate our approach by using a total of 60 cervical vertebrae. The objective is to find ROI and localize cervical spine centers. Thus, an automatic approach is proposed which gives generalization to traction therapy. Image analysis of C-spine is achieved with an accuracy of 98 %. Image characterized as normal or abnormal cervical image; also, exact traction weight is calculated using physics behind traction. 8% of the body weight will be the ideal weight to start traction with minimal side effect and highest therapeutic efficacy. Therefore, clinicians could adopt this weight as minimum weight in managing neck disorder requiring traction and goes up to the weight as calculated by proposed algorithm.

KEY WORDS: HOUGH TRANSFORMS, SPINAL TRACTION, SPECTRAL TRANSFORMATION, INTERVERTEBRAL POSTERIOR HEIGHT.

INTRODUCTION

In this paper, a smart approach is discussed to detect and localize changes in x-ray images of the cervical spine. The method discussed here for the treatment of cervical pain is a combination of image Processing and Physics theory, this analysis in future bears a major aid to doctors and will save considerable time. The goal of this research is to evaluate biomechanical efficacy and to reduce the range of the traction force (25lbs-4lbs) used for the treatment of cervical spinal traction by analyzing spinal structure. The study will help to improve outcomes, quality of care, and provide some standardization of treatment. For spinal column diagnosis the first step is vertebra detection.

This task becomes more problematic in case of the cervical X-ray images as these images are usually characterized by their low contrasts and noise due to skull bones. Preprocessing is done to locate and enhance the spine region of interest in x- ray images; this stage is usually

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followed by the processing stage which includes the boundary representation and segmentation algorithms based on feature vector extraction. Image processing has been used in all areas of research. It has become an important tool for visualization and analysis of data. In recent years, the field of medical imaging has required that the role of image processing to expand for better results (Huihao et al., 2019).

References till 2019-2020: In current scenario, back pain is a leading cause of work absenteeism. Ignorance of such pain becomes chronic disease which not only affect our spine, but also affect our work, afterwards it become major symptoms of disabilities. (Ehrlich et al., 1999; George E 2003). Three-dimensional imaging tests like MRI and CT scan, is ideal for analyzing& visualizing pathology of the IVD (intervertebral disc), neural structures such as the spinal cord. Imaging methods provide superior structural information and a better resolution (Schmitz et al., 2011); (Runshengwang et al., 2014); proposed a new method in which semiautomatic image analysis routine is used to analyze dendrite and synapse characteristics in immune-fluorescence images. Another method effective in reducing pain or improving intervertebral motions is HVLA-SM (High velocity low amplitude spinal manipulation) (Reed et al., 2015).

Another area of concerns is the cervical spine trauma which causes majority of spinal lesions. Many of these problems are the consequence of an abnormal spinal motion. A model of the human cervical spine was developed with a focus on accurate representation of the cervical spine using image processing algorithms. Models of each segment were constructed from the basic building blocks of the cervical spine: the intervertebral disc, the vertebrae, the ligaments, and the facet joints. The capsule ligament stiffness made an impact on segmental mobility and vertebral spatial position, and the sagittal angle of articular facet joint exerted an influence on disc pressure distribution. To predict effectiveness possibility of cervical traction the least absolute shrinkage and selection operator regression model was used to select potentially relevant features (Huihao et al., 2019; Yang et al., 2020).

The results of the study indicate that the no other algorithm at present using smart image processing algorithm for calculating exact traction weight in case of cervical spine. This research contribution is generalized traction weight calculation. The objective is to find ROI and localize cervical spine centers. The results of the study indicate that the no other algorithm is present using smart image processing algorithm for calculating exact traction weight in case of cervical spine. Individually in image processing algorithm the model was in reasonable agreement with the experimental data, and compared better than current models (Reed and Pickar, 2015).

MATERIAL AND METHODS

To find the accuracy of traction treatment a small survey was conducted under which data of 17 volunteer data and 12 X-ray image data were collected from 3 hospitals. In which 16 were males and 13 were females. Following observations are mention as: Traction force is not only dependent to the fractional part of body weight but also on several other factors which is not included in the main parameters for applying the traction force. For cervical traction the force applied i.e. 1/7th part of body weight and for lumbar traction the force applied i.e. 1/3rd of body weight is not practically implemented, but it varies according to age, gender, bone density, blood pressure, etc which is not included at present in the treatment. On the basis of survey conducted, the following data were recorded. The following data were taken from Narmada Trauma Centre (Bhopal), Rajiv Gandhi Hospital (Trilanga, Bhopal) and Peoples hospital (Bhanpur, Bhopal) (Yang et al., 2020).

Table 1. Survey Report of Some Patients Who WERE Going Under The Treatment of Traction

	Volunteer data base & the amount of traction weight applied			
Patient number (M/F)	Age of patient (in years)	Body Weight (in kg)	Type of traction	Applied Weight (in kg)
Patient 1 (M)	40	65	Cervical	7
Patient 2 (F)	38	55	Lumbar	20
Patient 3 (M)	33	55	Cervical	6
Patient 4 (M)	55	68	Cervical	8
Patient 5 (F)	22	58	Cervical	6
Patient 6 (F)	21	62	Cervical	6
Patient 7 (M)	38	52	Cervical	6
Patient 8 (F)	55	70	Cervical	8
Patient 9 (F)	49	75	Lumbar	25
Patient 10 (M)	39	90	Cervical	10
Patient 11 (F)	59	70	Cervical	5
Patient 12 (M)	50	75	Cervical	8
Patient 13 (M)	23	59	Cervical + Shoulder pain	8
Patient 14 (M)	40	60	Cervical	2
Patient 15 (M)	55	60	Lumbar	20
Patient 16 (M)	55	75	Lumbar	25
Patient 17 (M)	30	54	Cervical	7

Source: Narmada Trauma Centre (Bhopal), Rajiv Gandhi Hospital (Trilanga, Bhopal

This data leads to us to do work for the improvement of traction treatment. The proposed research is a smart approach in which cervical vertebra detection method using a statistical algorithm and template matching approach based on the Spectral Transformation technique. In this method an edge-based recognition process used to extract vertebra shapes and its invariance

to scale change, rotation and translation. This research is based on following steps:

Figure 1: Original X-RAY IMAGE OF C-Spine [SOURCE: PEOPLES HOSPITAL, BHOPAL]

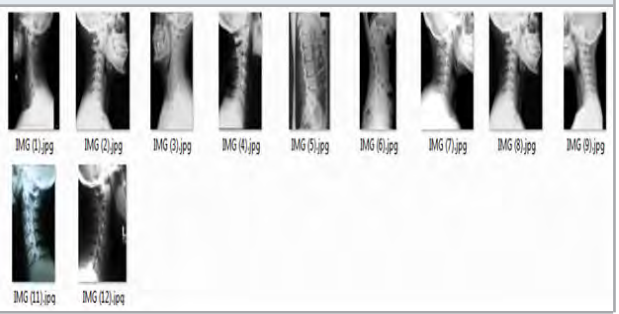
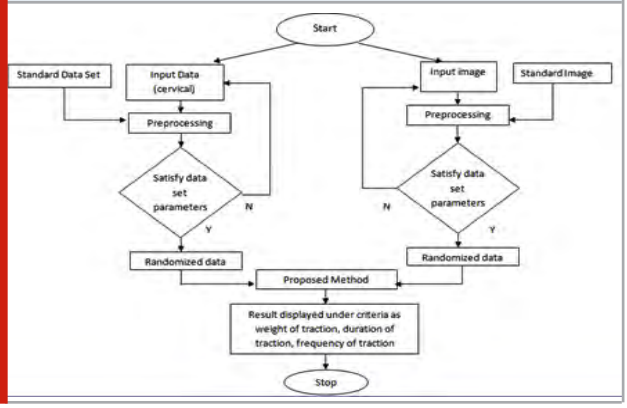


Figure 2: Flow chart for developing an algorithm for Spinal Traction



A. Modelling –modelling is aimed to make template of the vertebra, edge detection and to apply Hough transform.

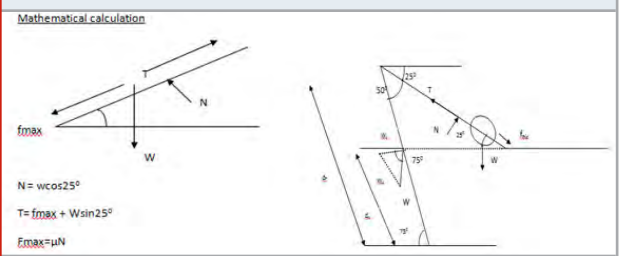
B. Centers detection-to enhance X-ray images histogram equalization is used as pre-processing. Canny and Sobel operators are used for edge detection and gradient computation.

C. Post-processing-In the last step adaptive filtering is used to find intervertebral distance.

D. Mathematical calculation-after determining the intervertebral distance, mathematics is applied to calculate traction weight.

Physics behind traction

Figure 3: free body diagram of traction machine setup



Below calculations are based on the traction machine setup, i.e. distance from ground, machine weight and total height. The cervical traction is applied to a patient; traction force is developed in backward while maintaining the system in equilibrium. Machine weight =15kg Center of mass height from ground= 102cm (max). If the distance from bottom to the center of mass is 102cm, then the total height from where force is applied is 173cm as distance from bottom to the center of mass 59% of total height.

From the free body diagram establishing momentum equation about the bottom

Ty.dT=Wy.dw Where: the distance from the bottom to the center of mass is

dW=59% of 1.73m =1.02m and dT= 85% Of 1.73m = 1.46mtherefore
Ty.dT=Wy.dw Tsin50^0.dT = Wy.dw, (fmax +wsin25^0) sin50^{0}x1.46 = Wcos75^{0}x1.02 As fmax = μ.N

In this paper for two body weight of a patient, calculations are done. Body weight is 70kg and 75kg, so head weight i.e. 8% of the total body weight are 5.6kg and 6kgs respectively. (μ.N + wsin25^0) sin500x 1.46 = Wcos75^{0}x1.02, Where N = Wcos25^0, here w is weight of the head of a patient

And the value of μ = 0.2, Now equating the values in the equation

(μ. wcos25^0 + wsin25^0) sin50^{0}x 1.46 = Wcos75^{0}x1.02
(0.2x5.6cos25^0+5.6sin25^0) sin50^{0}x 1.46 = Wcos75^{0}x1.02

The exact W i.e. traction weight can be calculated as

$$\frac{(0.2x5.6\cos25^0 + 5.6\sin25^0)\sin50^0x1.46}{(\cos75^0x1.02)}$$

The calculations have been coded and required results have been obtained.

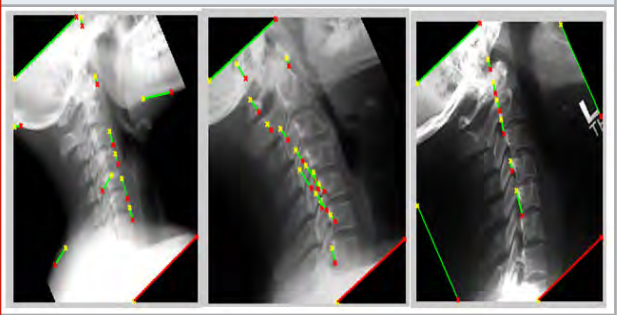
RESULTS AND DISCUSSION

In this research results have been calculated for radicular pain which occurs when the intervertebral separation is < 3mm. It has been suggested that the present research is not for patients with radiculopathy as it describes neurological condition. As the data set used in this research consists of X-ray images. The major problem faced while using X-Ray images are, the image data base is blurry, so vertebrae localization becomes the challenging task to perform manually. In order to enhance the image and to find region of interest image processing algorithm is used (Huihao et al., 2019).Next step is to resize the image [Fig-4].Longest segment has been detected using spectral transformation technique, in this research we use Hough transform for finding

longest segment [Fig-5]. Following operation is formed to get Region of Interest.

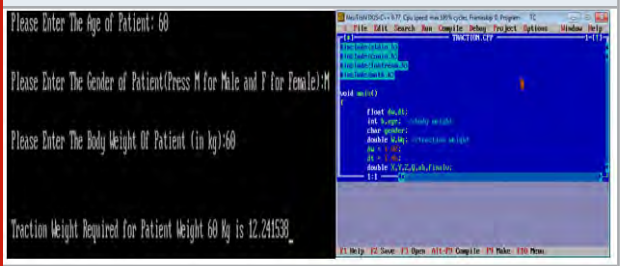
Figure 4: C-spine image for 3 cases [source: image data base collects from Peoples hospital, Bhopal]

Figure 5: Image transformations using spectral transformation [source: Processed image of the collected image database]



1. Morphological operations are performed now for boundary extraction [Fig-5].
2. Plot the contour of the spectrum.
3. In this step a matrix is formed according to brightness pixel value by applying condition BW1=1
4. By above step we get Region of interest (ROI) [Fig-6].

Figure 9: C++ code to detect traction weight based on body weight of patient under medical supervision for normal and abnormal images from image database collected from Peoples hospital, Bhopal.



After characterizing image as normal or abnormal image, an algorithm used to detect amount of traction weight required.

Table 3 shows the applied and proposed traction weights for the patient under medical supervision for neck disorder.

Figure 6: Initial segmentation stage [source: Processed image of the collected image database]

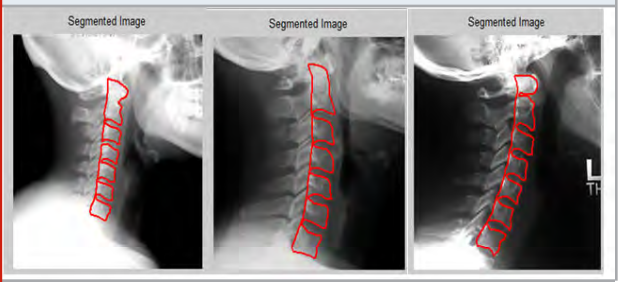


Figure 7: Locating ROI [source: Processed image of the collected image database]

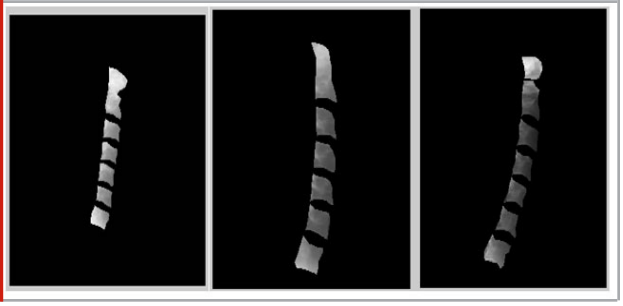
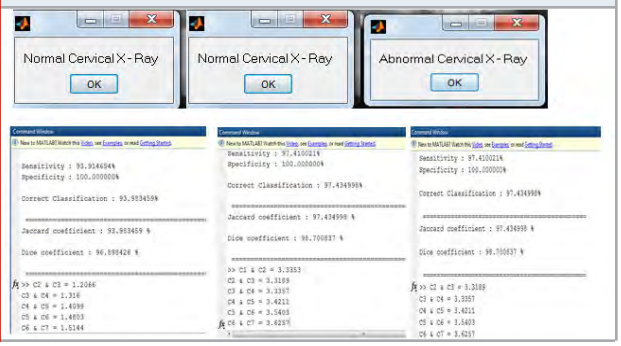


Figure 8: Intervertebral Posterior height calculated for three images from the image data base collected from Peoples Hospital, Bhopal



The intervertebral disc (IVD) is comprised of three distinct components: (a) the annulus fibrous, (b) the nucleus pulposus, and (c) the cartilaginous endplates. The annulus fibrous is the tissue of the intervertebral disc that surrounds the nucleus pulposus and forms the outer portion of the disc. Due to compressive load the posterior section of the intervertebral disc will undergo tension loading that causes the annulus to contract toward the center of the disc (White et al., 1990; Runshengwang and Ward, 2014). The intervertebral height for cervical spine is mentioned in the table given below.

Due to load, the intervertebral posterior height reduces. In this research, image processing algorithm is used to detect the intervertebral posterior height. As the study says for spinal traction relieves pressure on cervical spine, it can be done manually or mechanically. To get better results x-ray images of cervical area must be

studied along with the symptomatic problems. In this research both the symptoms as well as image analysis is performed in order to get better results (Fabian et al., 2012; Runshengwang and Ward, 2014). None of the study at present will work on reducing the range of traction force (25lbs-40lbs), as the amount of force applied will depends on the experience of physiotherapist. Most of the researches are using image data base of CT scan or MRI imaging but X-ray imaging is economic among the imaging technique used. 8% of the body weight will be the ideal weight to start traction with minimal side effect and highest therapeutic efficacy.

Table 3. Table shows the result of applied weight and proposed weight for C-spine traction. [source: Narmada Trauma Centre (Bhopal), Rajiv Gandhi Hospital (Trilanga, Bhopal)

Patient number (M/F)	Age of patient (in years)	Body Weight (in kg)	Type of traction	Applied Weight (in kg)	Weight calculated by algo(kg)	Weight calculated in lbs	Range of weight(max-min)(p<.0.5) in lbs
Patient 1 (M)	40	65	Cervical	7	13.26	29.23	26-32
Patient 2 (F)	38	55	Lumbar	20	NA	NA	NA
Patient 3 (M)	33	55	Cervical	6	11.22	24.75	22-27
Patient 4 (M)	55	65	Cervical	8	13.87	30.57	27.5-33.5
Patient 5 (F)	22	58	Cervical	6	11.83	26.08	23.5-28.5
Patient 6 (F)	21	62	Cervical	6	12.64	27.86	25-30.5
Patient 7 (M)	38	52	Cervical	6	10.60	23.36	21-25.5
Patient 8 (F)	55	70	Cervical	8	14.35	31.48	28-34.5
Patient 9 (F)	49	75	Lumbar	25	NA	NA	NA
Patient 10 (M)	39	90	Cervical	10	14.28	40.47	36-44
Patient 11 (F)	59	70	Cervical	5	15.30	31.48	28-34.5
Patient 12 (M)	50	75	Cervical	8	11.90	33.73	30-37
Patient 13 (M)	25	59	Cervical + Shoulder pain	8	13.26	26.23	23.5-28.5
Patient 14 (M)	40	60	Cervical	10	12.3	29.23	26-32
Patient 15 (M)	55	60	Lumbar	20	NA	NA	NA
Patient 16 (M)	55	75	Lumbar	25	NA	NA	NA
Patient 17 (M)	30	54	Cervical	7	11.02	24.29	21.5-26.5

Table 4. Accuracy Recognition

S.No	Study	IVD height	C23	C34	C45	C56
1.	Gilad & Nissan, 1986	Posterior	3.4mm	3.3mm	3.0mm	3.0mm
2.	Przybylski et al,1998	Posterior	3.4mm	3.4mm	3.7mm	3.9mm
3.	Present Research	Posterior	3.3mm	3.3mm	3.4mm	3.5mm
	Accuracy Recognition		97%	97%	98.5%	98.5%

Therefore, clinicians could adopt this weight as minimum weight in managing neck disorder requiring traction and goes up to the weight as calculated by proposed algorithm (Martiel et al., 2015).

CONCLUSION

In this paper, a new smart approach is used to detect C-spine vertebrae in X-ray images. A model of the human cervical spine was developed with a focus on accurate representation of the cervical spine using image processing algorithms. Models of each segment were constructed from the basic building blocks of the cervical spine: the intervertebral disc, the vertebrae, the ligaments, and the facet joints. This research contribution is generalized traction weight calculation. The objective is to find ROI and localize cervical spine centers. The results of the study indicate that the no other algorithm is present using smart image processing algorithm for calculating exact traction weight in case of cervical spine. Individually in image processing algorithm the model was in reasonable agreement with the experimental data, and compared better than current models.

Furthermore, it was recommended that the future goal of this model would be to implement so that a Clinical prediction has been developed which will improve decision-making and reduce time & cost. Thus, an automatic approach is proposed which gives generalization to traction therapy. Considering the advantages (feasible, accurate and economic) of the goal, if achieved, this may be used to develop traction machines in future which will be algorithm based and would work automatically on the basis of the factors to be considered while applying the therapy. Angle of head and table selected is 250, in future different angle settings will be done for C1-C2 & C6-C7.

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A Modern Equation for Determining the Dry-spell Resistance of Crops to Identify Suitable Seeds for the Breeding Program Using Modified Stress Tolerance Index (MSTI)

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ABSTRACT

Today's world is facing various challenges that impose remarkable stress on the very environment and species that man relies on for his existence. Drought, for instance, is one of the main sources of environmental stress. To tackle the environmental difficulties caused by drought, investigating types of plants with drought-resistant properties is both useful and, arguably, essential. The selection procedure of plant materials includes many formulas, such as SSI, STI, TOL, GMP, YI, YSI, and MP. Under drought stress and non-stress conditions, these formulas rely on seeds' weight. Low viability of seeds can mislead research with inauthentic data for the selection of an optimal species of plants. Thus, this study suggested the inclusion of 'seed viability' in these formulas. Based on the results of other research projects, the Stress Tolerance Index (STI) is the most useful selection criterion. Since breeders have to meet the needs of both research laboratories and the breeding program, selecting a set of seed with high viability is vital, hence the final formula: MSTI= ((Ypi xYsi)/Yp2)×SV Ysi= yield of the cultivar in stress condition, Ypi= yield of the cultivar in normal condition, Yp2= SV= seed viability. *Note: SV is for the selected condition that we want to use seeds in It should be noted that the provided formula here is only for the breeding program and laboratory experiments.

KEY WORDS: MSTI, DROUGHT STRESS TOLERANCE, NEW FORMULA, BREEDING PROGRAM.

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INTRODUCTION

The drought-induced stress is of the main factors that limit the production of crops, including wheat on the national and international levels. The limiting role of drought becomes more prominent when it comes to dry and semi-arid regions (Kirigwi & Van Ginkel et al., 2004). The severity of this issue becomes clearer when statistics indicate about 1/4 of Earth's land is dry, and an estimated 1/3 of the world's arable land suffers mild

to severe water shortage (Gholamin and Khayatnezhad 2020). Drought tolerance is defined as the ability of crops to grow and produce under conditions of water shortage. If the drought persists over the long term, the stress that it causes can negatively impact plants' metabolic reactions, physiological properties, unsettle the stages of growth, and damage soil's water storage capacity.

Crops indicate different drought tolerance in comparison with wild species. In reaction to a condition where access to water is severely limited, crops go through serious deficit-related trauma, perish, or significantly lose yield, whereas, in the wilderness, plants may fight the deficit and remarkably survive without yield decline. Nevertheless, breeders in dry areas, where land suffers a shortage of water, have always considered the drought-resistant properties of their crops a determining breeding factor, seeking ways to maximize production while keeping water consumption to the minimum, (Talebi, Fayaz et al., 2009).

While attempts at enhancing grain yield in areas with favorable environmental conditions have proven much successful, genetic modification of crops to maximize the yield in harsh environments remains a challenge to date (Richards, Rebetzke et al., 2002). Hall defines drought resistance as a genotype's relative yield compared with other genotypes' yield when subjected to the same level of drought stress (Hall 1993). Research studies often measure genotypes' drought susceptibility as a function of yield reduction under drought-induced stress (Blum 1988) whilst the values are confounded with genotypes' differential yield potential (Ramirez-Vallejo & Kelly, 1998) defined 'stress tolerance' as the different levels of yield under stress (Ys) and non-stress (Yp) conditions.

They also defined mean productivity (MP) as the average yield of Ys and Yp. Fischer and Maurer proposed a new index that could help distinguish the more susceptible plants from others in an experiment: It was called the 'stress susceptibility index' or SSI (Fischer & Maurer, 1978). A new advanced index, the Stress Tolerance Index, which is used for the identification of an optimal genotype with the highest production under stress and non-stress conditions, was introduced by '90s (Fernandez, 1992). The list of criteria used in determining drought resistance is not limited to STI, though, as other research studies use geometric mean (GM), mean productivity (MP), and tolerance (TOL) (Gholamin and Khayatnezhad 2020). Not all breeders use the geometric mean as only those interested in improving the relative performance tend to employ it. The reason for this tendency lies within the unstable nature of the stress caused by drought or harsh conditions as it can change in severity in the long run (Ramirez-Vallejo and Kelly 1998, Sallam et al 2019).

In a research study for evaluation of pattern variance of stress resistance in wheat genotypes, they found that SSI for genotypes and their ranking pattern varies every year (Clarke & DePauw et al., 1992). Studying spring wheat plants, Guttieri and his team found that an SSI value more than 1 meant an above-average susceptibility to the stress caused by drought (Guttieri & Stark et al., 2001). (Golabadi & Arzani et al., 2006), and (Sio-Se Mardeh & Ahmadi et al., 2006) proposed that high mean productivity (MP), geometric mean productivity (GMP), and the stress tolerance index (STI) can facilitate the selection of a drought-tolerant genotype under stressed and non-stressed conditions.

Breeders mainly seek new ways of selecting suitable genotypes with high resistance to drought-induced stress so that they can enrich their genotypes storage with new modified variations with improved performance under harsh conditions. (Clarke, DePauw et al., 1992). Other research studies in this field utilized different indexes for identifying the plant with optimal resistance to water deficit (Khayatnezhad and Gholamin 2010), however, but in breeding program and laboratory experiments we need alive seeds and seed viability is important than seed weight.

Thus, seed viability is included in the Stress Tolerance Index equation (Eq.1).

Eq. 1

$$MSTI= ((Ypi \times Ysi)/Yp2)\times SV/100$$

Ysi= yield of cultivar in stress condition, Ypi= yield of Ysi= yield of the cultivar in stress condition, Ypi= yield of the cultivar in normal condition, SV= seed viability in stress. *Note: SV is for the selected condition that we want to use seeds in. Depicted below, is the explanation of this new idea:

Based on the conventional methods, EXP 2 is more resistant than EXP1 (Table 1), so we can select this genotype for the food program because EXP 2 has more seeds, thus more useful. We measured the seed viability of both samples (Table 2).

The study conducted the measurement using the suggested formula:

$$MSTI= ((Ypi \times Ysi)/Yp2)\times SV/100$$

Table 1. Stress tolerance values for example

Genotype	Ys	Yp	SSI	STI	TOL	MP	GMP	YSI	YI
EXP1	60.55	82.3	2.69	0.53	21.75	112.57	70.59	0.74	0.69
EXP2	95.8	137.45	3.09	1.39	41.65	185.35	114.75	0.7	1.09

Ysi= yield of cultivar in stress condition, Ypi= yield of cultivar in normal condition, SV= seed viability in stress.
*Note: SV is for selected condition that we want to use seeds

Table 2. values of seed Viability in stress condition for examples

Genotype	SA
EXP1	91%
EXP2	65%

Table 3. Percentage of growth ability and Stress tolerance

Genotype	STI	MSTI	Percentage of growth ability and Stress tolerance
EXP1	0.53	0.482	90.56
EXP2	1.39	0.903	66.90

As depicted in Table.3, the impact of seed viability on STI's percentage of growth in EXP 1 and EXP 2 was respectively 90.56% and 66.90%. The results find EXP2 unsuitable for the breeding program seed. Also, the EXP2 genotype is only suitable for food, but EXP 1 is stronger than EXP 2. Therefore, this genotype had fewer 1000-seeds weight, which meant it wasn't a fitting candidate for food consumption, while it proved more suitable for the breeding program. Based on the findings of these studies below, it is proposed that these indexes are relatively limited in terms of selecting the optimal genotype: (Khayatnezhad & Hasanuzzaman et al., 2011), (Khayatnezhad, Khayatnezhad, & Gholamin 2012) (Khayatnezhad, Gholamin, & Khayatnezhad, 2010), (Gholamin et al., 2010), (Khayatnezhad & Zaeifizadeh et al., 2010), and (Khayatnezhad & Zaefizadeh et al., 2011).Since this study needs alive seeds for the breeding program and relevant experiments, it will select high-viable seeds. Hence, these two factors, drought-resistance, and seed viability can play a vital role in helping ensure successful experimental projects to maximize yields and minimize water consumption.

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Biological Control of Fusarium Wilt of *Cajanus cajan*

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ABSTRACT

Cajanus cajan (L.) Millsp. commonly called Pigeon Pea a leguminous plant grown extensively for food, feed, fodder, fuel also grown as an intercrop and in crop rotation to improve the fertility of the soil in sustainable organic farming. This plant gets infected by the pathogen *Fusarium udum* causing wilt disease which is one of the major constraints in the production and productivity of pigeon pea. This pathogen is reported throughout the world infecting *C. cajan*. The extensive use of chemical fungicides results in environmental pollution, the resistance of pathogens towards fungicides, hazardous to humans and animals. This necessitated the need to adopt eco-friendly and sustainable management of diseases, like using antagonistic fungi against the pathogen. In our present study, 15 different fungal isolates were isolated from different rhizosphere soil and used for Our present study involved the in vitro - dual culture assay as well as in vivo - greenhouse bioassay was performed to analyze the antifungal efficacy of antagonistic fungi against *F. udum*. The *in vitro* and *in vivo* investigations showed that *Cephalosporium acremonium*, *Lasiodiplodia pseudotheobromae, Penicillium frequentans* and *Epicoccum sorghinum* equally inhibited *F. udum* when compared to various *Trichoderma* spp. Also, under greenhouse conditions, the root length, shoot length, and the number of leaves of plants was found to be increased significantly ($p \leq 0.05$) in treatments with the talc-based biopesticide formulations of the antagonistic fungi. Hence these fungal isolates can also be used as a potential biocontrol agent for sustainable wilt diseases management caused by *Fusarium udum* and the extensive usage of chemical fungicides can be avoided to control the wilt disease of *Cajanus cajan*.

KEY WORDS: BIOCONTROL, EPICOCCUM SORGHINUM. FUSARIUM SP,, LASIODIPLODIA PSEUDOTHEOBROMAE, WILT DISEASE.

INTRODUCTION

Cajanus cajan is an important constituent in the category of pulses among all Indians due to the availability of 20-21% protein providing an energy-rich cereal diet. According to FAO statistics, this crop is cultivated in an area covering 4.6 million hectares globally and India accounted for about 73% of the global production in the year 2007. It is also an important crop of Karnataka contributing about 18% and 12% to total area and production respectively (GOI, Agricultural statistics, 2013). This protein-rich legume is prone to a multitude of diseases of which fungal diseases lead to a productivity loss of approximately 22 - 25 % each year amounting to a loss of up to Rs. 50,000 crores annually. Among the fungal diseases, *Fusarium* wilt disease caused by a soil-borne pathogen *Fusarium udum* (Fu) Butler is associated with extensive yield losses of pigeon pea in India. *Fusarium*

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species are common soil inhabitants that usually infect through roots and penetrates the vascular system of plants, causing wilt and substantially reducing yields due to their ability to survive in soil, plant roots, and dead plant debris for several years Saremi and Burgess (2000).

There is an increasing demand for controlling crop diseases in an eco-friendly way by using biological pesticides rather than the traditional way of using chemical pesticides. The extensive use of chemical pesticides poses a negative impact on the environment as well as humans. They also are toxic to normal microflora of the rhizosphere Molli et al, (2015). Therefore, the use of biocontrol agents is promising in protecting the rhizosphere or spermosphere by inhibiting the pathogen (Marx, 1972) and by competing with the pathogen for limiting the growth nutrients (Chet, 1979; Couteaudier and Alabouvette, 1990). Scientists have studied the mechanism of biological control by antagonistic fungi and bacteria to control plant pathogens extensively in the past two decades (Janisiewicz et al. 2000). In our present study, we explore the possibilities to isolate a potentially promising biocontrol agent against wilt disease of pigeon pea.

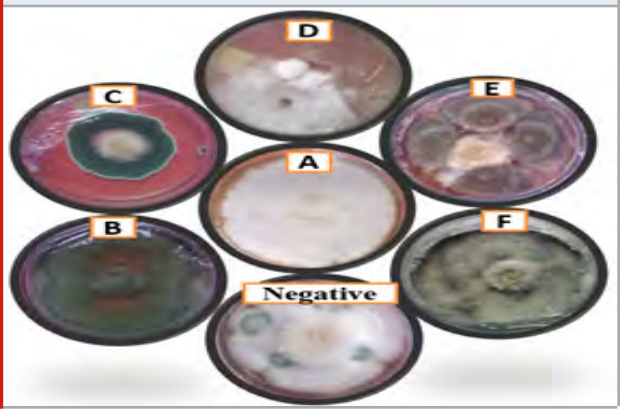
MATERIAL AND METHODS

For the isolation of pathogen from wilt infected *C. cajan*, the wilt infected plant samples were collected from the pigeon pea field of GKVK, Bangalore. Isolation of fungi from diseased root and leaves samples was done by standard tissue isolation method (Aneja, 2003). The isolated pathogen was subjected to pathogenicity assay by sick soil method to confirm the pathogenicity of the pathogen (Dehariya et al. 2015; Sumitra 2006); Nikam et al., 2011; Arunodhayam et al., 2014). The isolated pathogen was identified by morphological characteristics and the pure cultures of the pathogen were maintained on PDA slants and stored at 4oC (Leslie and Summerell, 1991).

Figure 1: Isolated Pure culture and Microscopic view of *Fusarium udum*

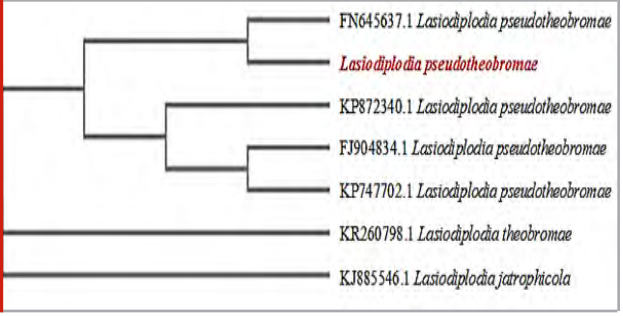
For the isolation of antagonistic fungi, rhizosphere soil of wilt infected pigeon pea was collected. The obtained samples were processed and plated by a serial dilution method and pour plate technique (Aneja, 2003). All the plates were incubated for 5- 7 days at 21-240C. The pure culture of the fungal isolates was prepared, and they were identified using morphological characters up to species level using the keys as proposed by Joseph Gilman (1950) and Barnett (1998). The isolates that were not able to identify through morphological characteristics were subjected to molecular characterization.

Figure 2: Dual culture method (Representative plates): A: control, B: *Trichoderma* sp.-isolate I, C: *Penicillium* frequentans D: *Lasiodiplodia pseudotheobromae* E: *Epicoccum sorghinum* F: *Cephalosporium acremonium*. Negative: *Penicillium frequentalis*.



For the molecular characterization of Isolate-1, the pure cultures of Isolate-1 were obtained by placing the hyphal tip of the mycelia aseptically on sterilized Potato Dextrose Broth and was incubated at 24oC for 6-7 days. The extraction of genomic DNA from the pure cultures of both the isolates was performed by – Cetyl trimethyl ammonium bromide (CTAB) method (Ausubel et al., 1994). The Polymerase chain reaction of the obtained DNA was performed with universal primers for fungal genome, namely ITS 1 - (5'-TCC GTA GGT GAA CCT GCC G-3') forward ITS 4 - (5'- TCC GCT TATTGATAT GC-3') reverse primers. These primers were obtained from Chromous Biotech Pvt. Ltd. Bangalore, India. ITS region of rDNA was visualized by UV trans-illumination (352 nm) and the expected DNA band was excised from the gel using a sterile scalpel and placed into a 1.5 ml microtube.

Figure 3: Cladogram of *Lasiodiplodia pseudotheobrome*



This DNA was purified using gel extraction kit (Chromous Biotech Pvt. Ltd. Bangalore, India) according to the manufacturer's specifications. The purified PCR product was sequenced at Chromous Biotech Pvt. Ltd.

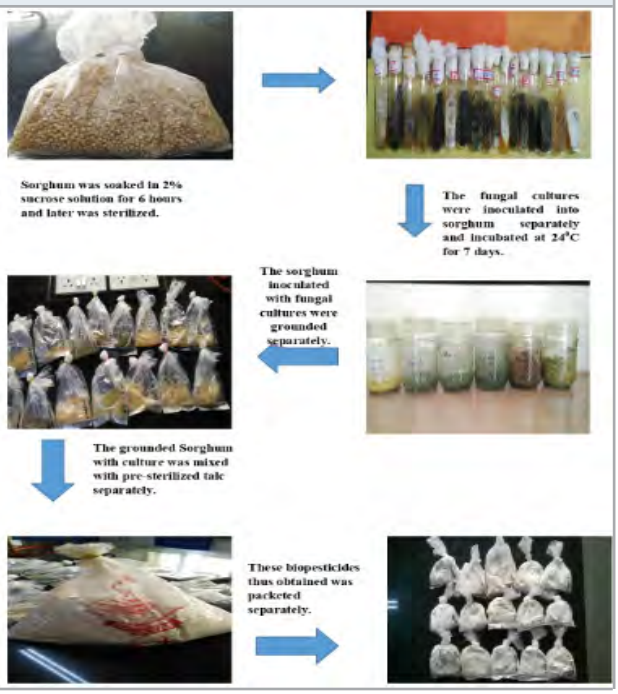
Bangalore, India. Sequences were determined by the chain termination method using an ABI 3130 Genetic Analyzer. Sequencing was done in the forward and reverse direction. The sequence was generated using data analysis software (Seq Analysis_ v 5.2). The obtained rDNA gene sequence was used to carry out BLAST with the database of the NCBI gene bank. Based on maximum identity scores first 10 sequences were selected and aligned using Multiple sequence alignment software program Clustal W2 to prepare Cladograms for the analyzed species. The rDNA sequence of an isolate was deposited in NCBI Genbank.

For the *in vitro* screening of antagonistic fungi against the pathogen, the dual culture method (Skidmore and Dickinson, 1976) was followed to analyze the degree of antagonism exhibited by 15 fungal isolates against the pathogen. The inoculated sets were incubated at 280C for 7 days. Triplicates of each test plate were maintained. The degree of antagonisms of each bio-agent against the test pathogen in dual culture was graded on a scale of 1-5 as proposed by Bell et al., (1982) and the percent inhibition of radial mycelial growth of the pathogen was calculated using the following formula given by Singh et al., (2002).

$$I = [(C-T)/C] \times 100$$

Where, I- the percent inhibition
C - colony diameter in the control plate and
T- the colony diameter of the pathogen

Figure 4: In vitro production of bio pesticides



For the preparation and biocontrol control studies of talc based biopesticides, antagonistic isolates, exhibiting a high degree of antagonism against the pathogen *in vitro* conditions, were chosen for preparing talc-based biopesticides. Solid substrate fermentation was followed for the production of talc-based biopesticide (Mukesh et al., 2015) (Fig 04). The efficacy of theprepared biopesticides was studied to control the wilt disease of pigeon pea. It was tested by sowing pigeon pea seeds into artificially made pathogen infested soil as described by (Bell et al., 1982). 2g each of the 12 prepared talc powder-based biopesticide was added to each pot containing pigeon pea plants with the inoculation of *F. udum* in triplicates. The number of leaves, root and, shoot length was measured in each pot along with negative and positive control and it was subjected to statistical analysis.

Figure 5: In vivo studies: A: Triplicates of Pigeon pea plant; B: Representative figure-Trichoderma viridae.

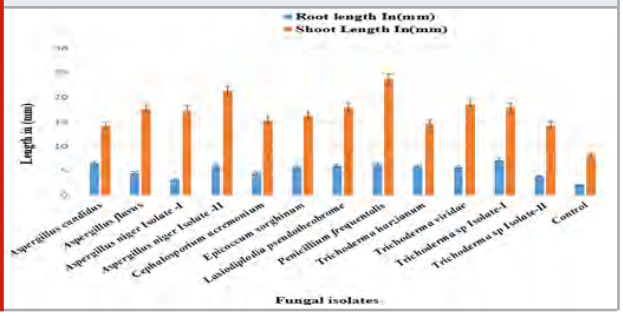
Table 1. Percent (%) Inhibition of growth of *Fusarium udum* and *Fusarium oxysporum* f. sp lycopersici by different fungal isolates and Grade Assessment for the interaction between the pathogen and antagonistic fungi

Antagonistic organisms	Grades	% inhibition of radial growth of fungi *Fusarium udum*
Aspergillus candidus	3	78.82±0.02^{i}
Aspergillus flavus	1	78.52±0.02^{j}
Aspergillus niger-isolate I	1	78.51±0.02^{f}
Aspergillus niger-isolate II	1	78.22±0.01^{g}
Cephalosporium Acremonium	1	88.22±0.02^{c}
Cladosporium sp.-isolate I	5	11±0.10l
Cladosporium sp-isolate II	4	33.33±0.01^{k}
Epicoccum sorghinum	1	72.65±0.00^{h}
Lasiodiplodiapseudotheobrome	1	83.76±0.01^{d}
Penicillium frequentalis	1	0±0.0^{m}
Penicillium frequentans	5	82.63±0.02^{e}
Trichoderma harzianum	1	89.66±0.01^{a}
Trichoderma sp.-isolate I	1	78.25±0.02^{g}
Trichoderma sp.-isolate II	1	88.52±0.02^{b}
Trichoderma viridae	1	83.74± 0.03^{d}

Means (n=3) in each column followed by the same letter are not significantly different (p<0.05) from each other according to Duncan's multiple range test (DMRT)

For the data collection and statistical analysis, the data of the results obtained from in vitro and in vivo studies were tabulated and these were subjected to statistical analysis by using MSTAT-C (Version 1.41) and Microsoft excel software. Analysis of Variance (ANOVA) of differences in the treatments and the least significance tests were carried out. Significance was evaluated at P<0.05 for the tests. For each factor analyzed, the means of the different treatments were compared and ranked using Duncan multiple range test at P < 0.05 using MSTAT-C (Version 1.41). The graphs were prepared using Microsoft Excel software.

Figure 6: Efficacy of fungal formulations on *Fusarium* wilt of *Cajanus cajan* (Pigeon pea) under greenhouse conditions (Root and shoot length)



RESULTS AND DISCUSSION

Pigeon pea plants exhibited symptoms of wilt disease showing mild chlorosis followed by leaf abscission before succumbing to the disease. The pathogen isolated was identified as *F. udum* (Fig 01) and its pathogenicity was proved by reisolating the pathogen from the artificially infected plants. Similarly, Dehariya et al., 2015 performed the pathogenicity assay for *F. udum* and successfully reisolated the pathogen from the diseased pigeon pea plant. Following this antagonistic fungal species were isolated from the rhizosphere soil samples were identified till species level based on morphological characters.

Table 2. Efficacy of fungal formulations on *Fusarium* wilt of *C. cajanunder* greenhouse conditions

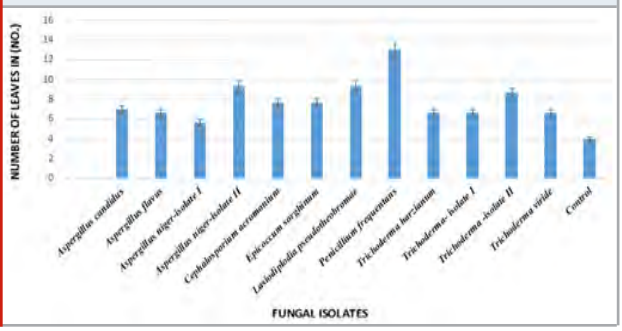
Biocontrol agents	Root length in (mm)	Shoot length in (mm)
Aspergillus niger Isolate-I	3.33±0.849837^{de}	17.33±1.69^{bc}
Cephalosporium acremonium	4.50±0.408248^{bcd}	15.33±0.47^{c}
Epicoccum sorghinum	5.83±1.545603^{abc}	16.33±1.88^{bc}
Aspergillus niger Isolate-II	5.93±2.192918^{abc}	21.33±6.34^{ab}
Lasiodiplodiapseudotheobrome	6.07±0.492161^{ab}	18.00±2.16^{bc}
Trichoderma harzianum	6±2.160247^{abc}	14.67±3.68^{c}
Penicillium frequentans	6.33±1.247219^{ab}	23.67±3.39^{a}
Trichoderma sp.Isolate-I	7.33±1.649916^{a}	18.00±2.16^{bc}
Trichoderma sp.Isolate-II	4.00±0.816497^{cde}	14.33±4.92^{c}
Aspergillus flavus	4.6±0.432049^{bcd}	17.67±4.18^{bc}
Aspergillus candidus	6.67±1.545603^{ab}	14.23±3.02^{c}
Trichoderma viridae	5.83±0.849837^{abc}	18.67±0.47^{abc}
Control	2.23±0.20548^{e}	8.37±0.26^{d}

Means (n=3) in each column followed by the same letter are not significantly different (p<0.05) from each other according to Duncan's multiple range test (DMRT)

Isolate-1 which could not be identified based on morphological characters was subjected to molecular characterization for precise identification of organism till species level (Pushpa et al., 2014). Isolate-1was identified as Lasiodiplodia pseudotheobromae as it showed 99% similarity with the sequence of L. pseudotheobroma deposited in NCBI- Genbank with the accession numbers - KP747702.1, KP872340.1, FN645637.1and FJ904834.1. The phylogenetic position confirms that our isolate corresponds to *L. pseudotheobromae* and that it would have been evolved from *L. theobromae* as per the cladogram obtained (Fig 03). The isolate was deposited in Genbank with the accession number: KX289694.1.Dual culture assay (Fig 02, Table 01) and Duncan's Multiple Range Test showed that 12 fungal isolatesi.e., *T. viridae, T. harzianum, Trichoderma* sp.-isolate I, *Trichoderma* sp.-isolate II, *L. pseudotheobromae, E. sorghinum, A. niger*-isolate 1, *A. niger*-isolate II, *A. flavus, A. candidus, P. frequentans, C. acremonium* displaying class-1 and positive antagonismwith percent of growth inhibition in the range of 72.66%-89.66%.While, *P. frequentalis, Cladosporium* sp.-isolate I, *Cladosporium* sp.-isolate II showed poor inhibition rates of 33.33%, 11% and 0% respectivelydisplaying class- 5 negative antagonism.

Greenhouse experiments revealed that *A. candidus, A. flavus, Trichoderma* sp. isolate-I on infected plants showed a moderate growth of root, shoot and number of leaves. Whereas, *A. niger*-isolate I, II, *C. acremonium, E. sorghinum, L. pseudotheobrome, P. frequentans, T.harzianum Trichoderma* sp. -isolate-II,T. viridaeshowed highest growth of root and shoot while it showed moderate growth of number of leaves (Fig 05, 06, 07; Table 02).

Figure 7: Efficacy of fungal formulations on *Fusarium* wilt of *Cajanus cajan* (pigeon pea) under greenhouse conditions (Number of leaves).



It can be concluded that the antagonistic organisms have an ability to produce zones of inhibition, which can be attributed for the production of various secondary metabolites. Similar studies have been reported by various investigators for the production of zones of inhibition by antagonistic fungi around the pathogen and the increasing concentrations of antifungal and fungistatic metabolites lead the dominating antagonistic organisms to overgrow the pathogens, thereby suppressing their growth (Royse and Ries, 1977; Odigie and Ikotun, 1982). Several scientists have worked on the in vitro screening of different strains of Trichoderma viride and *T. harzianum* against *F. udumand* proved significant inhibition of growth of various isolates of *F.udum* (Patel et al, 2011).

CONCLUSION

The use of fungi as biological control agents has found its deserving slot in the sustainable management of a spectrum of fungal and bacterial diseases in recent years. The use of biocontrol agents to combat fungal diseases has been magnified recently, due to the increasing negative environmental impact of hazardous chemical pesticides. The present investigation provides a detailed insight on the use of biological means for managing the wilt pathogen of *C. cajan* caused by *F. udum* both in vitro and in vivo. Though moderate growth inhibition activity was exhibited by *L. pseudotheobrome* and *E. sorghinum*, it was found that they could vigorously grow and overtake or suppress the growth of both the pathogens similar to that exhibited by all *Trichoderma* sp. which is referred to as hyper parasitism. These evidences prove that the wilt disease could be considerably reduced by the use of talc formulations of fungal antagonists. And also, that biocontrol agents other than Trichoderma sp. can be scaled-up for talc-based formulations and mass production of fungal biopesticides for controlling *Fusarium* wilt diseases of *C. cajan*.

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The Prevalence of Extreme Severity of Autoaggression Among Residents of Russia

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ABSTRACT

Suicide in Russia is not uncommon. This requires careful consideration of its prevalence and factors contributing to it among Russians. This can effectively adjust the measures taken in Russia to prevent suicide, and will also help in the discussion by representatives of various sciences of the maximum number of aspects of the problem that should be taken into account when developing new measures to reduce the suicide rate in Russia. The material for this research was the information sources contained in the open press. The research methods in the work were the methods of analysis and synthesis, induction and deduction and the method of mathematical processing. The highest levels of suicide per 100 thousand population were noted in five Russian federal districts: North-West, Volga, Ural, Siberian and Far East. At the same time, the mortality rate of the population from deliberate autoaggression with lethal self-harm was lower than the national average in three federal districts: Central, South, North Caucasian. Most often, suicides in Russia are committed by single people and divorced people. People with a high level of education are less susceptible to suicide than people with low education. Suicides in Russia are dominated by persons of low social status. It was found that the most common reason for suicide in Russia is the loss of social status. In addition, the causes of suicide can be progressive illness and economic problems. A suicide attempt in Russia is often promoted by a state of drug or alcohol intoxication. Suicide for Russia remains a serious problem, which can be solved largely by optimizing social and economic conditions, as well as carrying out systematic preventive work with the most threatened by suicide contingents of the population.

KEY WORDS: SUICIDE, BEHAVIOR, LIFE, LAW, AUTOAGGRESSION.

INTRODUCTION

The steadily developing medical and biological science continues to form a reliable foundation for the well-being of modern mankind (Vorobyeva et al., 2018).

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Much attention continues to be paid to the disclosure of the mechanisms of the human brain functioning and the connection between its work and social behavior (Bespalov et al., 2018). At the same time, modern man continues to face a host of various medical and social problems. In many ways, they are associated with the lack of various means, the continuing impossibility of a complete cure for many diseases, limited resources available, frequent shortages of food and the presence of various forms of deviant behavior in a part of the population (Polskaya, 2015).

One of its variants is autoaggression, an extreme form of which is considered suicidal behavior, which has recently become a global public health problem in many

countries. Modern researchers consider suicide to be a conscious refusal of a person from life associated with actions aimed at stopping it and classify it as the most extreme form of deviant behavior. It is now customary to call suicide deliberate actions that led to death, and actions as a result of which an attempt was made to deprive oneself of life, but due to various circumstances, it did not result in a fatal outcome. In this regard, recently, researchers began to distinguish between fatal (completed) suicide and non-fatal suicide (or suicidal attempt) (Ananyin, 2020).

According to available information, suicide rates in many countries, including Russia, remain high. This situation attracts a large number of researchers in order to clarify its various aspects. Due to the high mortality rate from suicide, Russia continues to suffer significant social and economic losses. This can be reflected at the level of the gross national product, inhibiting economic growth (Sergienko et al., 2007). In view of the negative statistics on the level of suicide, Russia continues to actively work to prevent suicide among the population. In this regard, the continuation of the analysis and assessment of the level of prevalence of this phenomenon among the population of different regions of Russia is of particular relevance. This can effectively adjust the measures taken in Russia to prevent suicide, and will also help in the discussion by representatives of various sciences of the maximum number of aspects of the problem that should be taken into account when developing new measures to reduce the suicide rate in Russia, (Zharkov and Protsik, 2016).The purpose of the present work is to examine the level of prevalence of extreme severity of autoaggression among Russians.

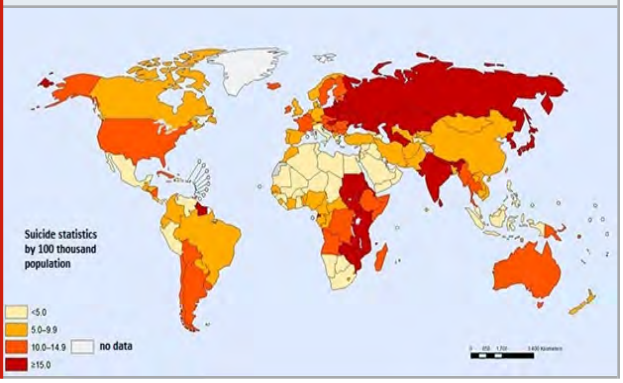
MATERIAL AND METHODS

The material for this research was the information sources contained in the open press. The search was carried out in the database of the scientific electronic library eLIBRARY.RU and in the Scopus database. The research methods in this work were the methods of analysis and synthesis, induction and deduction and the method of mathematical processing using standard statistical programs (Novikov and Novikov, 2010).

RESULTS AND DISCUSSION

Suicide occurs in all countries of the world, affecting people of all nations, cultures, religions, genders and classes. Available statistics show that the countries with the highest suicide rates in the world are very diverse. Unfortunately, Russia occupies one of the leading positions in these statistics (according to preliminary data for 2020, there are 31 suicides per 100 thousand), experiencing a gradual increase. According to preliminary data, in 2020, she entered the top five leaders in this indicator, along with Lithuania (31.9 suicides per 100 thousand), Guyana (29.2 suicides per 100 thousand), South Korea (26.9 suicides per 100 thousand)., Belarus (26.2 suicides per 100 thousand) (https://www.yaplakal.com/forum3/topic2124734.html). The prevalence of suicides among the world's population is shown in Figure 1.

Figure 1: The prevalence of suicides on the planet (https://avatars.mds.yandex.net/get-en_doc/192582/pub_5cc0519c569af600b33b5f49_5cc052716c165100b0a54183/scale_1200)



When analyzing open official sources of the Ministry of Health of the Russian Federation, the mortality rate from intentional self-harm was 13.8 cases per 100 thousand population in 2017 and reached 13.3% of all external causes of death. The study of the territorial characteristics of mortality of the population from intentional self-harm (suicide) in 2017 made it possible to identify Russian federal districts with levels above or below the national average (Table 1):

Table 1. Suicide rates by districts of Russia in 2017

Federal districts of Russia	The number of suicides per 100 thousand population
Northwest	14,2
Privolzhsky	16,7
Ural	15,6
Siberian	23,7
Far Eastern	18,2
Central	10,0
Southern	8,4
North Caucasian	4,3

It turned out that the highest suicide rates per 100 thousand of the population were noted in 2017 in five Russian federal districts: North-West, Volga, Ural, Siberian and Far East. At the same time, in 2017, the mortality rate of the population from deliberate autoaggression with fatal self-harm (suicide) was lower than the national average in three federal districts: Central, South, North Caucasian.Analyzing these indicators for the constituent entities of Russia included in all federal districts, significant differences were also established. In the Siberian Federal District, the highest suicide rate per 100 thousand population was found in

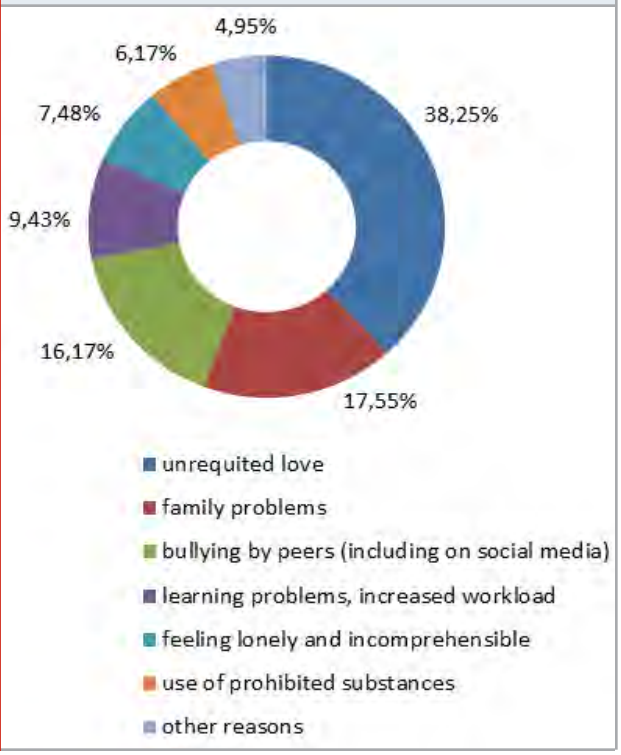
the Republic of Buryatia (39.6), and the lowest in the Republic of Tyva (5.6). In the Far Eastern Federal District, the maximum mortality rate from deliberate self-harm was recorded in the Jewish Autonomous Region (41.7), and the minimum - in the Khabarovsk Territory (0.5). In the Volga Federal District, the highest and lowest level of the studied indicator per 100 thousand of the population was, respectively, in the Udmurt Republic (33.9) and in the Samara region (4.0). In the Urals Federal District, the highest suicide rate per 100 thousand population was found in the Kurgan Region (35.8) and the lowest in the Khanty-Mansi Autonomous Okrug (8.4).

In the Northwestern Federal District, the maximum mortality rate of the population from intentional self-harm was registered in the Nenets Autonomous District (43.2), and the minimum - in the Murmansk Region (5.7). In the Central Federal District, the highest and lowest level of the studied indicator per 100 thousand of the population was noted, respectively, in the Ivanovo region (21.5) and in the city of Moscow (2.7). In the Southern Federal District, the highest suicide rate per 100 thousand population was found in the Republic of Kalmykia (17.7), and the lowest in the Astrakhan region (0.4). The lowest mortality rates of the population from intentional self-harm in Russia in 2017 were established in the North Caucasus Federal District. At the same time, the highest and the lowest level of the studied indicator per 100 thousand of the population was in this region, respectively, in the Karachay-Cherkess Republic (9.4) and in the Chechen Republic (0.6) (Russia in numbers, 2018).

The age-specific mortality rates of the population from intentional self-harm in the Russian Federation in 2017 per 100 thousand of the population were: 1.5 at 10-14 years old, 8.4 at 15-19 years old, 14.0 at 20-24 years old, 16.4 25-29 years old, 18.9 at 30-34 years old, 19.8 at 35-39 years old, 18.7 at 40-44 years old, 17.6 at 45-49 years old, 16.3 at 50-54 years old, 15.5 at 55-59 years old, 13.4 at 60-64 years old, 13.9 at 65-69 years old, 15.3 at 70-74 years old, 20.9 at 75-79 years old, 20.3 at 80 -84 years old, 27.6 years old and older.In general, in the Russian Federation, the death rate from suicide per 100 thousand of the population in 2017 in the age group 0-17 years was 1.3, in the working age - 17.8, in the group over working age - 14.5. It is noteworthy that the highest suicide rate in the Russian Federation in 2017 per 100 thousand of the population of working age is noted in the age groups of 30-34 and 35-39 years old (Korolenko and Dmitrieva, 2015).

It was found that in Russia, conscripts (up to 70% of all suicides in the army occur in the first year of service), convicts (60% of all suicides occur during the first three months and in the last months before release) are at high risk groups (Crime, 2017). Also, a very negative situation is observed among adolescents. A number of cities in Russia are covered by "epidemics" of teenage suicides. According to available data in Russia, the suicide rate among young people is 53 cases per 100 thousand population (Ignatenko, 2017). So, in 2016, the number of suicides increased by 57% compared to 2015 and amounted to 720 cases (Zotina et al., 2013). The reasons for teenage suicides in Russia are not very diverse (Nesvat, 2016). Their spectrum is shown in Figure 2 below.

Figure 2: Reasons for attempted suicide among adolescents in Russia.



Comprehending the results obtained in the study, it must be said that suicidal behavior is an activity in which obvious elements of self-destruction are necessarily present: from expressed aloud thoughts of suicide to explicit actions to physically destroy oneself. Suicidal behavior among the population of Russia is found in the form of completed suicide, suicidal attempts (attempts) and intentions (ideas). These forms are usually viewed as stages or manifestations of the suicide phenomenon. Suicidal tendencies in Russia encompass suicidal intentions, thoughts and frequent statements about unwillingness to live. A very important characteristic is considered in Russia the suicidal indicator - a value reflecting the prevalence of suicide cases among the population or a separate social group (Bardenstein et al., 2011; Korolenko and Shpiks, 2012).

The suicidal actions of Russians, as a rule, are not their impulsive reaction to a separate violation of their mental state. Observations show that suicide among Russians is a decision that has arisen over a long period of time against the background of serious personal or family difficulties. Moreover, such an attempt itself is often impulsive. The total mass of Russian suicides includes three main categories: people with severe mental disorders, patients with borderline mental disorders, and completely healthy people (Borisonik and Kholmogorova, 2018). At the

same time, all Russian suicides have obvious objective and subjective manifestations of socio-psychological disorders. The presence of this maladjustment can be manifested by a violation of the behavior of a Russian in his social environment, a weak ability to optimally cope with existing social phenomena or with pathological changes in his behavior (Aminov, 2014).

Researchers believe that subjective impairment is manifested by a number of negative changes - from negative psychological experiences to the obvious manifestation of psychopathological actions. In the course of the progression of psychological maladjustment in Russians, pre-suicidal and suicidal stages are formed. For the transition from the pre-suicidal stage to the suicidal one, Russians always have a suicidal conflict. In the regions of Russia where suicides are most common, such conflicts are very often formed with a low resistance of part of the population to stress. This is seen as an important prerequisite for suicidal behavior among Russians and can be caused by many reasons. Despite a number of reasons, in any case, this conflict is very real for a person, accompanied by painful experiences and a desire to get rid of them. The second phase of the conflict - suicidal, is an illogical attempt to eliminate this conflict through self-destruction of a person (Igntenko, 2017).

In the light of the above, suicidal behavior among Russians should be associated with an unsuccessful combination of environmental, personal and psychopathological moments (in the case of a person's mental disorder progression). As a result of the emerging situation, a person experiences suicidal actions and a complete suicide may occur, which is an external form of suicidal behavior. Fortunately, among Russians, the ratio of suicide attempts and completed suicides is 10: 1 (Korolenko and Shpiks, 2012; Zotina et al., 2013).

It is noted that the number of suicides committed among Russian men is higher than among women. This can be explained that men consider the motives and reasons for suicidal actions more seriously. They perceive personal and family problems (unfair treatment on the part of relatives and others, divorce and family conflicts, an obstacle to meeting an urgent need, prolonged loneliness, unsuccessful love, little attention from everyone around them) are perceived as more hopeless circumstances and are considered as strong motives for suicide. In the presence of mental health, men may have more pronounced internal conflicts, often disrupting the state of physical health (somatic diseases, physical suffering); the consequences of any antisocial acts of a suicide (fear of legal liability, fear of punishment or shame); conflicts in the educational or professional sphere (insolvency, failure in school or at work, decline in prestige, unfair demands), as well as the presence of acute domestic difficulties (Sukhareva, 2017).

Due to the widespread prevalence of suicides in Russia, preventive measures are being taken at the local, regional and federal levels. To prevent suicides in Russia, anti-crisis services are being created that work around the clock. A serious component of the preventive actions of these services is the active identification of suicidal persons among Russians and targeted psycho-corrective work with them in order to prevent suicide. The generally recognized risk factors for suicide among Russians are social isolation, especially the loss of connection with family and friends, mental disorders, a previous suicide attempt, drug and alcohol use (Sergienko et al., 2007; Aminov, 2014).

CONCLUSION

Suicidal behavior in Russia is conditioned by three important components: - individual personality traits, the situation of socio-psychological conflict and the degree of adaptation of the personality to it. It has been established that most often suicides in Russia are committed by single people and divorced people. Russians with higher education are less susceptible to suicide than those in Russia with low and incomplete secondary education. Suicides in Russia are dominated by persons with a relatively low social status (workers, unemployed, unemployed, and non-students). A common reason for making a decision to commit suicide in Russia is the loss of social status (first of all, dismissal). Also, frequent causes of suicide among Russians can be a progressive illness, as well as economic problems leading to the difficulty of acquiring housing, clothing, and food. At the same time, in Russia, the proportion of deaths in 2017 from suicide were at the time of death in a state of drug intoxication (33.4%) and 92.6% in a state of alcoholic intoxication.

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Modulatory Role of Riboceine and Vitamin C Supplementation on Altered Oxidative Stress Status of Alloxan-induced Diabetic Wistar Rats

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ABSTRACT

This study examined the anti-oxidative effects of Riboceine and vitamin C supplementation in the amelioration of tissue damage that results from oxidative stressors in diabetic rats. Twenty-five (25) mature Wistar rats (150-180kg) were used for the study. They were randomly assigned into five (5) groups of five rats each: Normal Control (NC), Diabetic Mellitus(DM) (induced by Alloxan at 120mg/kg body weight i.p), Diabetic rats treated with 30mg/kg body weight of RiboCeine (DM+RibCys), Diabetic rats treated with 125 mg/kg body weight of Vitamin C (DM+VitC) and Diabetic rats treated with 30 mg/kg body weight of RiboCeine + 125mg/kg body weight of Vitamin C (DM+RibCys +VitC). Following period of 20-day treatment, all the rats were sacrificed and samples of pancreas, kidney and liver tissues were collected and assayed for changes in oxidative stress biomarkers: Glutathione (GSH), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), Glutathione s-transferase (GST) and Malondialdehyde (MDA) levels. The tissues were also examined for Histo-architectural changes. Data were analysed using One Way Analysis of Variance (ANOVA) followed by Fisher's Least Significant Difference (LSD). A p-value of $p < 0.05$ was considered statistically significant. RibCys and DM+RibCys+VitC treated rats, showed statistically significant decrease in hyperglycaemia. However, RibCys dosed rats, showed no statistically significant effect. The DM rats showed statistically significant increased MDA and decreased CAT, SOD, GPx, GST levels in the tissues. The RibCys, VitC and DM+VitC+RibCys treated rats, showed statistically significant amelioration in increased MDA and decreased CAT, SOD, GPx, GST levels. Present results concluded that RibCys has ameliorative effects on diabetes induced-oxidative stress. Also, RibCys, VitC and VitC+RibCys have equally antioxidative effects on tissues characterized by biomarkers of oxidative stress.

KEY WORDS: RIBOCEINE, VITAMIN C, DIABETES MELLITUS, OXIDATIVE STRESS, ALLOXAN, ANTIOXIDANTS.

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INTRODUCTION

Diabetes mellitus (DM) describes a metabolic disorder with complex pathophysiology and multiple aetiology characterised by chronic hyperglycaemia and disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin action, or both leading to lethal consequences in patients (Yu et al, 2020). Free radical-mediated destruction of pancreatic beta cells

(site of insulin production) has been suggested as a major process that promotes pancreatic beta cells destruction in the pathophysiology of DM (Sundaram et al., 2006). In people with diabetes, glucose levels build up in the blood and urine, causing excessive urination, thirst, and hunger problems with fat and protein metabolism (Puhr et al, 2020).

Antioxidants plays vital role to maintain and improve health, protect cells, tissues and organs in living organisms after routine anabolic and catabolic processes. However, the therapeutic efficacy of antioxidants such as glutathione has been found to be negatively affected by aging, stress, insomnia, and exposures to toxic chemicals, with adequate rest as a key accelerator of an increase in its levels (Ghasemi-Dehnoo et al, 2020). The roles of anti-oxidants in scavenging and checking the excesses of free radicals in dying tissues and the molecular basis linking oxidative stress to Diabetes mellitus has been well established (Matkovics et al, 2007; Habib et al, 2020).

Riboceine consists of a scientific blend of D-Ribose and L-Cysteine compund, which makes its discovery a landmark that was not born by chance (Esposito and Giuglano, 2002). This ground breaking compound is a demand-release nutrient, activated by the body's cells. Riboceine enters the bloodstream and is then used by the body to produce glutathione, the body's master antioxidant, and ATP, the cell's natural fuel and energy source (Adelakun et al, 2018). Riboceine has been clinically and scientifically proven as the only supplement that can effectively deliver cysteine into the cell, enabling it to produce optimal amounts of glutathione (Nagasawa et al., 2004). The anti-oxidant mechanism of tissue regeneration is generic and applies also to riboceine (Benedict et al, 2017). However, scanty literatures exist on the anti-oxidant effect of Riboceine and possible mechanism of potentiation of those effects especially when administered with vitamin C on diabetic animals. Therefore, the rationale of this study is targeted at devising a measure towards understanding the modulatory role of riboceine and vitamin C supplementation on altered oxidative stress status in alloxan induced diabetic Wistar rats.

MATERIAL AND METHODS

Animal Handling and Procurement: Twenty-five (n = 25) mature Wistar rats were used for this study. They were procured from the Laboratory Animal Center of the Faculty of Basic Medical Sciences, Delta State University, Abraka. The animals weighed between 120g-200g and were housed in well-ventilated animal unit provided by the Department of Pharmacology, Delta State University, Abraka, Delta State. The animals were acclimatized for a week with standard environmental condition (12 hrs. light/dark cycles). During the entire period of study, animals were fed with standard grower mash diet (Composition of the grower's marsh) and water ad libitum, in a standard wire meshed wooden cages for 7days prior to commencement of the experiment.

Induction of Diabetes Mellitus: A 65mg of Alloxan monohydrate (Sigma-Aldrich, U.S.A.) was dissolved in saline and administered intraperitoneally into fasted rats at a dose of 120mg/kg body wt. The solution was fresh and prepared just prior to the administration. The animals were considered diabetic if the blood glucose values of the overnight fasted rats were > 200 mg/dl on the third day, following Alloxan injection (Yashwant et al., 2011). Glucose levels were tested by using One Touch Ultra Mini Glucometer (Accu-Check, Roche, Germany) with a drop of blood obtained by tail vein puncture (Osinubi et al., 2018).

Study Design and Grouping: Twenty-five (25) mature Wistar rats were used for this study. They were randomly divided into five (5) groups of five (n=5) rats each. The rats were sacrificed after 20 days of treatment. Below is a breakdown of the treatment protocol for each group:Group A = Normal Control (NC) rats, fed standard rat diets and water ad libitum, Group B = Diabetic (DM) rats, fed standard rat diets and water ad libitum,Group C = Diabetic rats treated with 30mg/kg body weight of RiboCeine (RibCys) daily, Group D = Diabetic rats treated with 125 mg/kg body weight of Vitamin C (VitC) daily, Group E = Diabetic rats treated with 30 mg/kg body weight of RiboCeine + 125 mg/kg body weight of Vitamin C.

Drug Procurement and Administration: Riboceine (capsules) was purchased from a subsidiary of Max international, Benin, Edo state. Riboceine capsule (125mg/capsule) was dissolved in distilled water to form solution, at a standard dose of 30mg/kg (Babatunde et al., 2017) and administered orally once daily via an orogastric canula. Vitamin C (Ascorbic acid) tablet (100mg/tablet) were also purchased from pharmacy in Benin, Edo state. The tablets were dissolved in distilled water to form solution, at a standard dose of 125mg/kg (Shivavedi et al., 2014) and administered orally via an orogastric canula. The drugs were administered once daily, between 8:00am and 11:00am.

Samples Collection: All animals were sacrificed at the end of the study (20 days) by decapitation under light ether anesthesia. The kidney, liver and pancreas were taken out, washed, crushed and homogenized in potassium chloride (KCl) solution. The homogenate was diluted and centrifuged, with supernatant decanted and examined for antioxidant and antioxidant enzyme activities of Glutathione (GSH), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), and Malondialdehyde (MDA).Also, the kidney, pancreas and liver were sectioned for histological examination.

Biochemical Investigations: The tissues sample were homogenized in 50mM phosphate buffered saline (pH 7.4) by using a glass homogenizer. Half of the homogenates were centrifuged at 1000 g for 10 min at 4˚C to separate nuclei and unbroken cells. The pellet was discarded and a portion of supernatant was again centrifuged at 12,000g for 20mins to obtain post-mitochondrial supernatant. In homogenate, TBARS and GSH levels were estimated.

In post-mitochondrial supernatant, SOD, CAT and GPx activities were measured as described below:

Determination of Malondialdehyde (MDA) Assay: A TBARS assay kit (ZeptoMetrix) was used to measure the lipid peroxidation products, MDA equivalents. One hundred microliters of homogenate was mixed with 2.5ml reaction buffer (provided by the kit) and heated at 95˚C for 60min. After the mixture had cooled, the absorbance of the supernatant will be measured at 532nm using a spectrophotometer. The lipid peroxidation products will be expressed in terms of nmoles MDA/mg protein using molar extinction coefficient of MDA-thiobarbituric chromophore (1.56×105/M/cm) (Ohkawa et al., 1979).

Determination of GSH Assay: The GSH levels were measured using the method described by Sedlak and Lindsay (Sedlak and Lindsay, 1968). Homogenate will be mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in one centimetre quarts cells.

Determination of SOD Assay: The activity of SOD in the tissues was estimated using the method described by Kono (Kono, 1978), with the aid of nitrobluetetrazolium as the indicator. Superoxide anions are generated by the oxidation of hydroxylamine hydrochloride and are measured using spectrophotometer at 560nm under aerobic conditions. Addition of superoxide dismutase inhibits the reduction of nitrobluetetrazolium and the extent of inhibition is taken as a measure of enzyme activity. The SOD activity will be expressed as units/mg protein.

Determination of CAT Assay: The CAT activity was measured by the method of Aebi (1974), using hydrogen peroxide as substrate in post-mitochondrial supernatant. The hydrogen peroxide decomposition by catalase was monitored on spectrophotometer by following the decrease in absorbance at 240nm. The activity of enzyme will be expressed as units of decomposed/min/mg proteins by using molar extinction coefficient of H2O2 (71/M/cm).

Determination of GPx Assay: The enzymatic activities of GPx was determined by using ELISA kits in the post mitochondrial supernatant of colon homogenate on ELISA reader by following the manufacturer's instructions (Flohe and Gunzler 1984).

Histological Analysis of Tissues: After the animals were euthanized, pancreatic, liver and kidney tissues were carefully harvested and placed inside a well labelled tissue embedding cassette. The tissues were processed with a 24hours automatic tissue processor, contained in 12beakers i.e. 10glass beakers and 2 thermostatically controlled electric metal beakers containing paraffin wax. After the tissues had been processed, they were embedded with an automatic embedding mole containing paraffin wax. The paraffin wax became solidified (upon cooling) and formed a solid support medium for the tissue during sectioning. A microtome was used in cutting sections of the tissues. The sections cut was placed in grease free slides which were then placed on a hot plate for 30minutes in order for sections to adhere to the slides. Using the Haematoxylin and Eosin stains, the sections were dewaxed in xylene, taken to water with descending grades of alcohol i.e. from absolute alcohol to 95% alcohol, 70% alcohol and then water. Final outcome was mounted and viewed under the microscope (Siddalingaswamy et al., 2011).

Ethical Considerations: Ethical approval was sought and obtained (REC/FBMS/DELSU/19/64) from the Research and Bio-Ethics Committee of the College of Health Sciences, Delta State University, Abraka, Delta State.

Statistical Analysis: All data generated were subjected to statistical analysis using SPSS version 23 software IBM. This was done using analysis of variance (ANOVA) and least significance difference (LSD) test at p-values of less than 0.05 that is, at 95% confidence level ($p<0.05$). Values were reported as Mean ± Standard error of mean (SEM).

RESULTS

Table 4.1 showed the percentage change value outcome of the body weight, we observed highly statistically significant decrease in percentage change value of body weight in DMC group, when compared with NC group. Slight statistically significant increase percentage change value of body weight in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group.

Table 4.1: Percentage Change (%) Body Weight (g)

Sample Parameter	Initial	Final	% Change
NC	143.80±1.50	152.00±1.14	5.39±0.95
DMC	179.40±5.50	159.80±10.11	-13.53±0.89 **0.004
*DM+RibCys	165.80±3.84	167.80±3.68	1.13±1.93 *0.023
DM+VitC	159.60±8.00	160.60±9.99	-0.37±0.34 *0.035
DM+Rib Cys+VitC	161.00±8.02	163.20±5.96	1.46±0.28 *0.018

*significantly different at $p<0.05$, **$p<0.01$. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C

Table 4.2 depicted the percentage change value in fasting blood glucose level on day 5, 10 and 20, post treatment. On day 5, we observed slightly statistically

significant increase in percentage change value of fasting blood sugar in DM+VitC and DM+RibCys+VitC groups, when compared with NC group. Also observed was a statistically significant decrease in percentage change value of fasting blood sugar in DM+RibCys group, when compared with DM+RibCys+VitC group. On day 10, the results showed slightly statistically significant increase in percentage change value of fasting blood sugar in DMC, DM+ RibCys and DM+RibCys+VitC groups, when compared with NC. Also, slightly statistically significant decrease in percentage change value of fasting blood sugar in DM+ VitC group was observed, when compared with DM+RibCys group, and slightly statistically significant increase in percentage change value of fasting blood sugar in DM+RibCys+VitC group, when compared with DM+ VitC group was also observed. Results from day 20, showed slightly statistically significant increase percentage change value of fasting blood sugar in DM+ RibCys and DM+RibCys+VitC groups, when compared with DMC group.

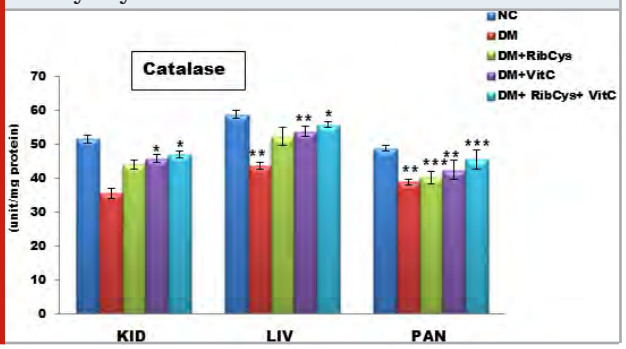
Table 4.2: Percentage (%) Change in Fasting Blood Glucose Level (mg/dl)

Sample Parameter	Day5	Day10	Day20
NC	0.20±0.74	-5.29±8.07	8.35±5.36
DMC	36.86±21.48b	39.56±15.14 *0.022	35.51±9.50
DM+Rib Cys	25.88±20.51 *0.028	53.94±13.10 **0.004	64.63±9.29 *0.040
DM+VitC	45.09±11.30 *0.044	13.73±17.80 *0.038	28.04±15.08
DM+Rib Cys+VitC	75.30±0.82 **0.002	57.31±5.79 **0.002 *0.026(D)	65.43±2.66 *0.035

*significantly different at p<0.05, **p<0.01, ***p<0.001. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C

*significantly different at p<0.05, **p<0.01, ***p<0.001. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C.

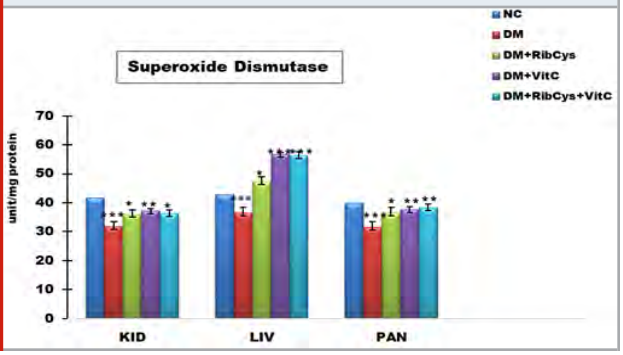
Figure 1: Tissue level of antioxidant enzyme (Catalase) activities in kidney, liver and pancreas of the rats after twenty days of treatment.



Changes in tissue levels of catalase (CAT) are presented in figure 1. CAT activity in kidney was statistically significantly higher in DM+VitC and DM+RibCys+VitC groups, compared with DM+RibCys group. Statistically significantly lower level of CAT liver and pancreas tissue was observed in DMC group, when compared with NC group. The DM+RibCys, DM+VitC and DM+RibCys+VitC groups, showed statistically significantly higher CAT activity in the liver and pancreas tissues, when compared with DMC group. Also, statistically significantly (*p = 0.022) higher CAT activity was observed in DM+VitC group, when compared with DM+RibCys group. The DM+RibCys+VitC group, showed statistically significantly (**p=0.006, **p=0.001) higher CAT activity in the pancreas tissue, when compared with DM+RibCys and DM+VitC groups respectively.

*significantly different at p<0.05, **p<0.01, ***p<0.001. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C

Figure 2: Tissue level of antioxidant enzyme (Superoxide Dismutase) activities in kidney, liver and pancreas of the rats after twenty days of treatment.



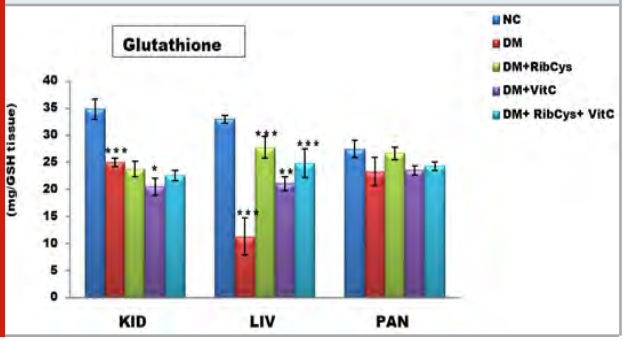
Changes in tissue levels of Superoxide Dismutase (SOD) are presented in figure 2. SOD activity in kidney was statistically significantly lower in DMC group, when compared NC group. Statistically significantly higher level of SOD activity in kidney tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. In the liver tissue, SOD activity was observed to be statistically significantly lower in DMC group, when compared NC group. Also, statistically significantly higher level of SOD activity in kidney and pancreas tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. The DM+RibCys group, showed lower statistically significant SOD activity in the liver, when compared with DM+VitC (***p=0.000) and DM+RibCys+VitC (***p=0.000) groups respectively. SOD activity in pancreas was statistically significantly lower in DMC group, when compared NC group.

Changes in tissue levels of Glutathione (GSH) are presented in figure 3. GSH activity in kidney was statistically significantly lower in DMC group, when compared NC group. Statistically significantly higher level of GSH activity in kidney tissue was observed in DM+VitC, when compared with DMC group. A

Statistically significantly higher level of GSH activity in liver tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. The DM+VitC (*p=0.047) group, showed higher statistically significant GSH activity in the liver, when compared with DM+RibCys group.

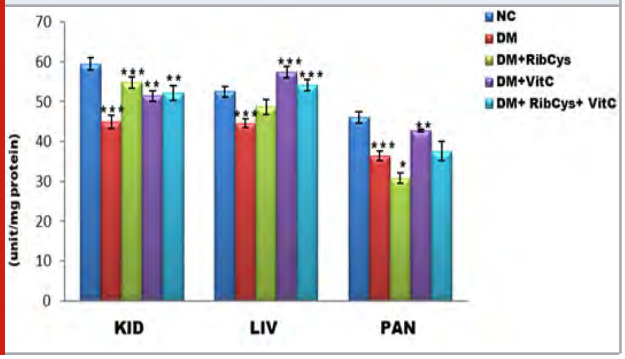
*significantly different at p<0.05, **p<0.01, ***p<0.001. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C

Figure 3: Tissue level of antioxidant enzyme (Glutathione) activities in kidney, liver and pancreas of the rats after twenty days of treatment.



*significantly different at p<0.05, **p<0.01, ***p<0.001. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C

Figure 4: Tissue level of antioxidant enzyme (Glutathione Peroxidase) activities in kidney, liver and pancreas of the rats after twenty days of treatment.

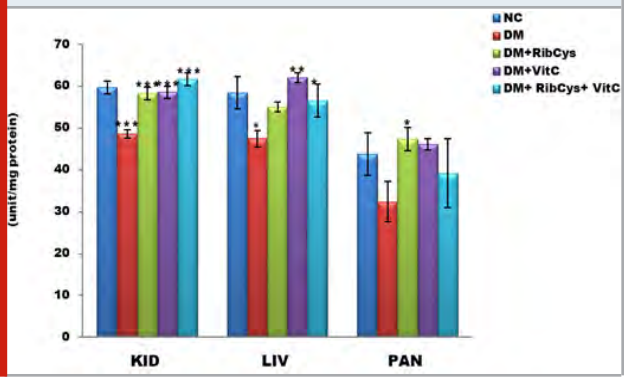


Changes in tissue levels of Glutathione Peroxidase (GPx) are presented in Figure 4. GPx activity in kidney and pancreas tissues were statistically significantly lower in DMC group, when compared NC group. A Statistically significantly higher level of GPx activity in kidney tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. GPx activity in liver was statistically significantly lower in DMC group, when compared NC group. While a Statistically significantly higher level of GPx activity in liver tissue was observed in DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. The DM+RibCys group, showed lower statistically significant GPx activity in the liver, when compared with DM+VitC (***p=0.000) and DM+RibCys+VitC (***p=0.014) groups respectively.

A Statistically significantly higher level of GPx activity in pancreas tissue was observed in DM+RibCys and DM+VitC groups, when compared with DMC group. The DM+RibCys group, showed lower statistically significant GPx activity in the pancreas, when compared with DM+VitC (***p=0.000) and DM+RibCys+VitC (**p=0.004) groups respectively. Also, DM+RibCys+VitC (*p=0.022) group, was observed to show higher statistically significant GPx activity in the pancreas, when compared with DM+VitC group.

*significantly different at p<0.05, **p<0.01, ***p<0.001NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C.

Figure 5: Tissue level of antioxidant enzyme (Glutathione s-transferase) activities in kidney, liver and pancreas of the rats after twenty days of treatment.



Changes in tissue levels of Glutathione Peroxidase (GST) are presented in Figure 5. GST activity in kidney was statistically significantly lower in DMC group, when compared NC group. A Statistically significantly higher level of GST activity in kidney tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. GST activity in liver was statistically significantly lower in DMC group, when compared NC group. While a Statistically significantly higher level of GST activity in liver tissue was observed in DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. A Statistically significantly higher level of GST activity in pancreas tissue was observed in DM+RibCys group, when compared with DMC group.

Changes in tissue levels of Malondialdehyde (MDA) are presented in figure 6. MDA activity in kidney was statistically significantly higher in DMC group, when compared with NC group. Statistically significantly lower level of MDA activity in kidney tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. The DM+RibCys group, showed lower statistically significant MDA activity in the kidney, when compared with DM+VitC (***p=0.000) and DM+RibCys+VitC (*p=0.031) groups respectively. Also, DM+RibCys+VitC (*p=0.025) group, was observed to show lower statistically significant MDA activity in the kidney, when compared with DM+VitC group.

The MDA activity in liver was observed to be statistically significantly higher in DMC group, when compared with NC group. Statistically significantly lower level of MDA activity in liver tissue was observed in DM+RibCys, DM+VitC and DM+RibCys+VitC groups, when compared with DMC group. MDA activity in pancreas was statistically significantly higher in DMC group, when compared NC group. While a Statistically significantly lower level of MDA activity in pancreas tissue was observed in DM+ RibCys and DM+RibCys+VitC groups, when compared with DMC group. Also, DM+RibCys (*p=0.026) group, was observed to show lower statistically significant MDA activity in the pancreas, when compared with DM+VitC group, and DM+RibCys+VitC (**p=0.008) group, was observed to have lower statistically significant MDA activity in the pancreas, when compared with DM+VitC group.

*significantly different at p<0.05, **p<0.01, ***p<0.001. NC: Normal Control; DM: Diabetic Mellitus; RibCys: Riboceine and VitC: Vitamin C

Figure 6: Tissue level of antioxidant enzyme (Malondialdehyde) activities in kidney, liver and pancreas of the rats after twenty days of treatment.

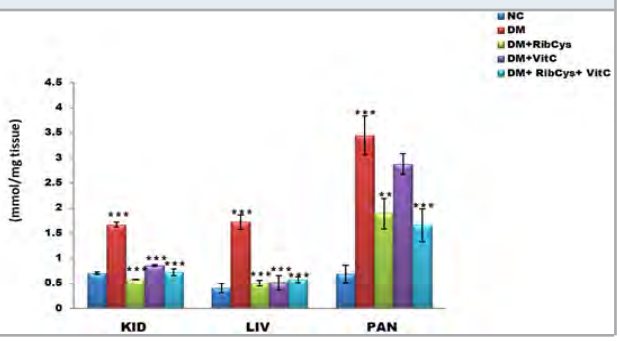
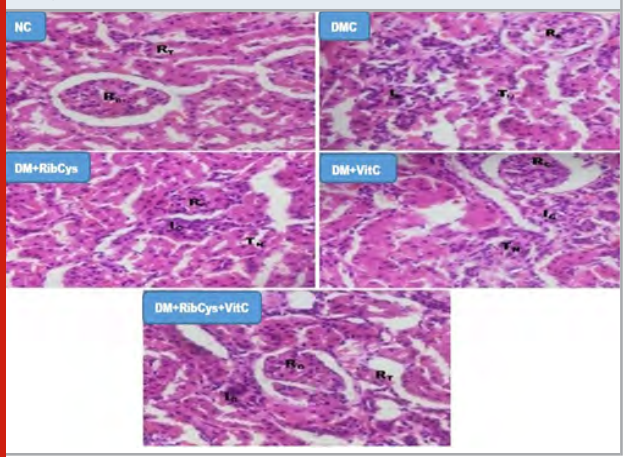


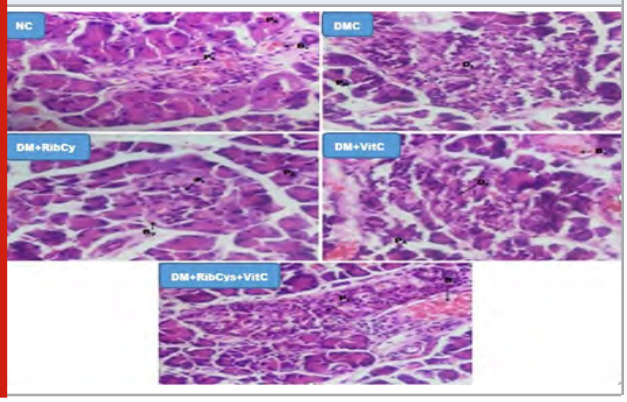
Figure 7: H&E stained kidney micrographs of NC, DM, DM+RibCys, DM+VitC and DM+RibCys+VitC groups, taken on 5 µm thick paraffin wax-embedded sections with a magnification of × 400.



Histological Changes:

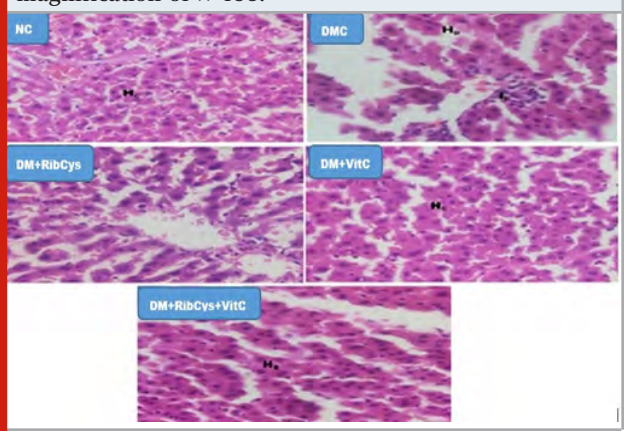
NC group, showed normal Renal corpuscles (R_C) and Renal tubules (R_C). DMC group, showed abnormal Renal corpuscles (R_C), tubular necrosis (T_N) and infiltration of inflammatory cells (I_C). DM+RibCys group, showed abnormal Renal corpuscles (R_C), mild tubular necrosis (T_T) and mild infiltration of inflammatory cells (I_C). DM+VitC group, showed normal Renal corpuscles (R_C), tubular necrosis (TN) and mild infiltration of inflammatory cells (I_C). DM+RibCys+VitC group, showed normal Renal corpuscles (R_C), normal Renal tubules (R_T) and mild infiltration of inflammatory cells (I_C).

Figure 8: H&E stained Pancreas micrographs of NC, DMC, DM+RibCys, DM+VitC and DM+RibCys+VitC groups, taken on 5 µm thick paraffin wax-embedded sections with a magnification of × 400.



Showing normal Pancreatic Islet (P_I), Pancreatic acini (P_A) and Blood vessels (Bv) in NC group. DMC group, showed normal Pancreatic acini (P_A) and Diabetic Islets (D_I): infiltration of inflammatory cells and vascular degeneration. DM+RibCys group, showed normal Pancreatic acini (P_A), mild infiltration of inflammatory cells(P_I) and mild vascular degeneration (Bv). DM+VitC group, showed normal Pancreatic acini (P_A), mild infiltration of inflammatory cells (D_I) and mild vascular degeneration (Bv). DM+RibCys+VitC group, showed normal Pancreatic Islet (P_I), Pancreatic acini (P_A) and Blood Vessels (B_V).

Figure 9: H&E stained Liver micrographs of NC, DMC, DM+RibCys, DM+VitC and DM+RibCys+VitC groups, taken on 5 µm thick paraffin wax-embedded sections with a magnification of × 400.



Showing normal Hepatocytes (HC) in NC group. DM group showed showing infiltration of Inflammatory cell (IC). DM+RibCys group, showed normal Hepatocytes (HC). DM+VitC group, showed normal Hepatocytes (HC). DM+RibCys+VitC group, showed normal Hepatocytes (HC).

DISCUSSION

The present study revealed a statistically significant percentage weight reduction in DM group (-13.53%) when compared to NC group (5.39%). This finding is in agreement with the finding of Ghada, (2013). The observed weight loss may be as a result of diabetes (Evcimen et al., 2004), leading to excessive breakdown of tissue proteins (Chatterjee and Shinde, 2002), as well as muscle wasting, dehydration and catabolism of fats (Hakim et al., 1997). Administration of Riboceine, vitamin C and riboceine + vitamin C to DM+RibCys (1.13%), DM+VitC (-0.37%) and DM+RibCys+VitC (1.46%) groups, showed statistically significant percentage weight increase when compared with DM group (-13.53%). The observed weight increase suggests interruption of the previously mentioned metabolic derangements. This observation agrees with the findings of Osinubi et al. (2018), who observed that riboceine maintained the body weights of the animals by protecting them from the cytotoxic effects of streptozotocin.

Data from our study showed that blood glucose level was increased in alloxan-induced diabetic rats, which may have resulted from the effects of alloxan which been proven to cause massive reduction in insulin release by the destruction of beta-cells of Islet of Langerhans and hence, inducing hyperglycemia (Ravikumar et al., 2010). In our study, there was decrease in the blood glucose level in DM, DM+RibCys, DM+VitC and DM+RibCys+VitC groups after twenty days of treatment (Table 4.2). This observation is corroborated by the research findings of Dawud et al. (2012), who reported that oral administration of antioxidant micronutrients and vitamins resulted in a significant decrease in the levels of blood glucose. Also, this finding, agrees with the finding of Osinubi et al. (2018), who reported a statistically significant decrease in the blood glucose level in diabetic rats treated with riboceine.

The present study has revealed that diabetes is linked with reduced activity of GST, GPx, GSH, Superoxide dismutase and lower levels of Catalase activities in kidney, liver and pancreas tissues of the diabetic rats compared with the control group. This observation agrees with the findings of Osinubi et al. (2018), wherein they observed a statistically significant decrease in CAT and SOD activities in diabetic rats compared with normal control rats. This findings agree with the finding of Yu et al., (2006); Arora et al., (2008); Cui et al., (2008). The decreased CAT activity in diabetes might reduce protection against free radicals (Alsaif, 2009; Soto et al., 2010). The results of this study, showed statistically significant increase in the levels of CAT and SOD activities in kidney, liver and pancreas tissues of the Riboceine, vitamin C and combination of riboceine + vitamin C treated rats, when compared with diabetic rats. This finding is corroborated by the findings of Osinubi et al. (2018). This observation may have resulted from the free radicals scavenging activities of CAT and SOD.

The findings of the present study, showed a statistically significant decrease in the levels of GSH, GPx and GST activities in the kidney, liver and pancreas tissues of the diabetic rats compared with the normal control group. Only in the pancreas, was statistically significant decrease was not observed. This reduction may have possibly resulted from the overwhelming effect of the free radicals on these antioxidants enzymes. Present study showed statistically significant increase in GR activity in liver, when DM+VitC and RibCys+VitC were compared with DM group. However, increase and decrease in GSH activities in pancreas and kidney were observed, when Vit C, RibCys and combined treatment groups were compared with DM group, but not statistically significant. The observation that showed ameliorative effect of RibCys or/and VitC in the tissues, agrees with the findings of (Maher and Al-Enazi, 2014; Kader et al, 2020), who reported that an increase in the activity of this enzyme, might have been mediated by GSH regeneration.

Protracted hyperglycemia may result in glucose toxicity and increase in ROS activity as well as increased lipid peroxidation especially in the pancreas which lacks sufficient antioxidants (Li and Leung, 2017). This is in tandem with the findings of this present study, which showed statistically significant increase in MDA activities in blood, pancreas, liver and kidney, in diabetic rats when compared with the normal control group. This observation agrees with the finding of Osinubi et al. (2018) suggesting indirect evidence of high free radical production in diabetes. The kidney, liver and pancreas of DM+VitC, DM+RibCys and DM+ RibCys+VitC treated groups, showed a statistically significant decrease in the activities of MDA, when compared with diabetic group. This finding is corroborated by the reported findings of Osinubi et al. (2018), Chuku and Chinaka, (2020).

We therefore speculate that the observed concomitant ameliorative effect in tissues of the animals that were treated with DM+RibCys and those treated with DM+VitC and DM+RibCys+VitC in the present study may have been influenced by the possibility of VitC and glutathione mutual sparing.The histopathological observations in the present work confirmed the improvement in treated groups when compared with the normal and diabetic control groups. The nephritic, hepatic and pancreatic tissue of diabetic rat demonstrated glomerular inflammation, tubular necrosis, inflammatory cells infiltration and vascular degeneration. The finding is in agreement with that of Elsner et al. (2002). The improvement of the histological structure in the treated groups suggest the anti-oxidative protective effects of riboceine and vitamin C, this observation agrees with the finds of Dawud et al. (2012) and Osinubi et al. (2018).

CONCLUSION

Weights of the diabetic and treated rats and change in the blood sugar of the VitC and VitC+RibCys treated rats were significantly decreased. The DM rats showed significant increase in MDA levels and decreased CAT, SOD, GPx, GST activities. Findings from the study revealed that RibCys, VitC and VitC+RibCys treated rats showed significant ameliorative effect on increased MDA levels and decreased CAT, SOD, GPx, GST activities and histological structures studied which suggest ameliorative effects on diabetes induced-oxidative stress.

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The Study of Path Analysis for Durum Wheat (*Triticum durum* Desf.) Yield Components

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ABSTRACT

This study used 10 durum wheat (*Triticum durum* Desf.) genotypes based from Azerbijan and Iran for experient purposes with an RCB design with four replications under non-stress full-irrigation and drought stress conditions in Ardabil, Iran during the 2015-2016 crop year. The study measured and evaluated the following parameters related to performance traits after harvest: tiller number, plant height, fertile tillers, length of main spike, peduncle length, number of internodes, awn length, spike original weight, number of seeds per main spike, TDW and grain weight. The results of variance analysis indicated that genotypes and their interactions with the environment differ significantly. Since the genotypic interaction with the environment was more significant, this study used genotypic correlation to study the relationship between variables. The comes about of relationship examination for the execution of the hereditary characteristics of the remaining edge relapse show detailed that the gather record (Hello there) had the most noteworthy affect, meaning it might be the basis for accomplishing the most elevated execution. TGW was The greatest coordinate negative impact on execution was related to the TGW.

KEY WORDS: GENETIC DIVERSITY, PATH ANALYSIS, PERFORMANCE, DURUM WHEAT.

INTRODUCTION

The world has been counting on wheat as an critical source of nourishment for thousands of years (Gholamin and Khayatnezhad 2020). Still, wheat, a whole-grain cereal, plays a vital role in feeding the ever-increasing population of the world. (Feldman 2000). On the international markets, durum wheat is usually traded at significantly higher prices than bread wheat. Besides, its great agronomic adaptability has caught the attention of many farmers. Also, it is extensively cultivated on a global scale in areas with suitable conditions for wheat farming. Arguably, durum wheat is a critical agronomic product and its farms are found in almost all continents: in the Mediterranean basin of Western Asia, Northern Africa, and South Europe (Khayatnezhad 2012, Khan, Alam et al. 2013).

Famously, dishes with durum wheat dough extend beyond geographical borders, as the art of cuisine in many countries has developed delicious, exclusive ways of using the grain (Gholamin and Khayatnezhad 2020. For instance, countries in North Africa and the Middle East cook traditional dishes like 'couscous' or 'bulgur', flat, unleavened bread; and in India, durum is consumed as 'chapatis .' Therefore, with such extensive use worldwide, it is no surprise that about 50% of world's production of durum wheat is converted into pasta products. It requires sufficient information of the relationship between the surrender and its component characters to design and implement an effective breeding program based on the yield components.

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In this respect, identifying the correlation coefficients between component characters is vital for choosing suitable materials for breeding (Afroz, Sharif et al. 2004). The path coefficient analysis, on the contrary, has proved more helpful in expounding the component-specific, coordinate and roundabout impacts on grain abdicate. Therefore, this study attempts to identify the relationships between abdicate and its component characters by assist analyzing the components causing such relations (Khan, Alam et al. 2013).

Environmental sources of stress, such as dryness and its associated consequences are critical, hindering factors of crop production, like wheat, on the national and international levels. The importance of this issue becomes more prominent in areas with moderate or high environmental stress related to dryness. (Kirigwi, Van Ginkel et al. 2004). The gravity of this issue becomes more palpable when we realize that about more than 1/4 of areas on land have dry climates, and 1/3 of the world's arable land is currently facing water deficit stresses (Kirigwi, Van Ginkel et al. 2004).

Some parts of the Mediterranean do not offer optimal levels of moisture conditions, thus hinder the optimal production of wheat. Technically, plants undergoing stressful presentation to dry spell within the vegetative stage have discernable disorders, such as leaf wilting, plant height decline, reducing within the number and zone of leaves, and delay within the precision of buds and blossoms (Talebi, Fayaz et al. 2009). By definition, drought tolerance is a plant's ability to grow and produce under water-shortage stress. If drought is remaining for the long term, the impacts of the related stress on a plant's metabolism are associated with its growth stage, SWSC (soil retention capacity) and physiology. In a relevant research on seven wheat cultivars from 53 lines, Moghaddam et al. observed significant differences in growth and other quantitative traits (Moghaddam et al., 2010).

They reported that while it took local cultivars longer to show the first indications of spike growth, they had higher heights and fewer grain seeds per spike and lower TGW, GY, and HI, compared with the newer cultivars. However, new cultivars and some local cultivars demonstrated similar yield traits. (Moghaddam, Ehdaie et al. 1997). A complicated quantitative trait, grain yield is impacted by several yield contributing characters (Xie 2015). The amount of genotypic diversity plays a vital role in developing enhanced crops with optimal yield traits under various agro-climatic conditions (Baye, Berihun et al. 2020). The correlations between genotypic and phenotypic traits are valuable indicators to determine the extent of the association of plants' various morpho-physiological properties with economic productivity. Despite not providing the relative significance of coordinate and circuitous impacts of growth and yield components, a correlation coefficient can be a useful tool in mapping the scope and heading of components' impact for the identification of main characters (Da Silva, Pedrozo et al. 2009).

Path analysis makes all that possible, with the introduction of the relationship coefficient for analyzed characteristics in the cultivars, the indirect and indirect impacts of those traits, and by presenting more authentic and reliable explanations of the causality between the studied traits. Relevant research studies refer to the positive relationship between grain abdicate and surrender component characteristics such as Hello there in wheat cultivars (Ghaderi, Zeinaali et al. 2009), biological yield (Kandic, Dodig et al. 2009), plant height (Leilah and Al-Khateeb 2005), grains per spike (Khan, Azam et al. 2010) and thousand kernels weight (Leilah and Al-Khateeb 2005). Recently, Ahmad et al., (2018) too talked about that grain yield's affiliations with the natural surrender, and the number of spikelets were genotypically and phenotypically positive and significantly noteworthy(Ahmad, Kumar et al. 2018). Path analysis calculates the quantitative direct and indirect impacts of component traits on grain yield (Rajput 2019).

Path analysis effectively divides the correlation coefficients into two main categories of direct and indirect impacts, explaining their associations in more details. (Majumder, Shamsuddin et al. 2008). Way investigation in this way licenses a basic examination of particular variables that deliver a given relationship and can be effectively utilized in defining an viable determination technique (Baye, Berihun et al. 2020). The aim of this research study is to investigate the genetic diversity in durum wheat cultivars, identify inherent traits beneficial for higher yields, and examine the correlation between these traits to select the best breed of durum wheat in terms of performance-related traits for cultivation under stress conditions, especially in Ardabil.

MATERIAL AND METHODS

For the sake of experiment, this study selected ten durum wheat Genotypes (Triticum durum Desf.) indigenous to Iran and the Azerbaijan due to their rumored contrasts in surrender execution beneath inundated and non-irrigated conditions (Table 1).

Table1. Name and Origon of used genotypes

Code	Genotype	Region
1	Hordeiforme (Miyaneh)	Iran
2	Leucurum (Qax)	Azerbaijan
3	Hordeiforme (Naxcivan)	Azerbaijan
4	leucumelan(Naxcivan)	Azerbaijan
5	Niloticum (Naxcivan)	Azerbaijan
6	Hordeiforme (Maragheh)	Iran
7	Leucurum (Sarab)	Iran
8	Leucurum (Tabriz)	Iran
9	Melanopus (Cheiltoxm)	Azerbaijan
10	Leucurum (Germi)	Iran

The study was conducted in the ARS at the Islamic Azad University of Ardabil in the 2015-2016 crop year. The university is located west of Ardabil, Iran. Based on the meteorological records in the last decade, the yearly precipitation is 310 mm and the normal annually temperature is 8.6°C within the try locale. Too, the normal least temperature is -22°C and the normal most extreme temperature is +30°C. The elevation is 1350 m over the sea-level. Seeds were hand bored and each genotype was sown in five lines of 1.5 m. The remove between the lines was 0.2m The test was conducted utilizing RCB plan with four replications. The Two levels of stretch medicines including: Full water system: characterized as assembly all the water needs of cultivars (100%) at distinctive development stages. Constrained water system: characterized as assembly water requests of the plant until the fertilization organize, and after that applying a organize water treatment until the terminal stage). Every line in 5 columns and 20 cm interims and 150 cm in width were planted. After manor, the try field was watered utilizing the spilling strategy to soak the soil and meet the water requests of cultivars and encourage germination.

The post-harvest performance-related measurements included the following traits and criteria: tiller number total, plant height, number of internodes, fertile tillers, awn length, peduncle length, spike original weight, length of main spike, TDW, number of seeds per main spike, and main spike, grain weight. The study used MATAT-C, SPSS 16, and PATH2 as data analysis tools. The study used the SMR method to conduct path analysis for performance based on the remaining characters. The collected data were analyzed in SPSS and the experiment used Duncan's MRT in 0.01% for comparing treatment means and the cluster method to divide the means into high and low yielding genotypes.

RESULTS AND DISCUSSION

As delineated in Table 2, the comes about of change examination shown that there were critical contrasts among the genotypes in terms of the larger part of characteristics, outlining their tall possibilities for breeding purposes. Since the characteristics were marginally and the environment moreover was noteworthy affect tradition, there this distinction was not startling (Manifesto, Schlatter et al. 2001).

Plant Height: As depicted in Table 2, the results of variance analysis demonstrated that the genotype, environment, and the interaction between them significantly impacted the plants' height (0.01). The mean comparison of the water treatment procedures revealed that full irrigation provided the best condition. As depicted in Table 3, Also, the mean comparison of genotypes concerning plant height indicated that 'genotype 4' had the highest and 'genotypes 2', and 'genotype 5' had the lowest height. Dividing the genotypes into different groups, the mean comparison of the interactions between the genotypes and the environment indicated that 'genotype 4' had the best interactive performance under stress conditions pointing to its optimal resistance in stress conditions. Conversely, 'genotype 2' had the worst interactive performance under similar harsh conditions. The results also appeared that genotypes with higher plant stature have a better push resistance compared to other genotypes. The results of the correlation analysis, presented in Table 4, reported the negative correlation (0.01) of the plant with HI, meaning an increase in the HI, caused the plant to decrease.

Main spike weight: Table 2 depicts the results of variance analysis, indicating that that the significant (0.01%) impact of genotype, environment, and the interaction between them for traits based on main spike weight. The cruel comparison demonstrated that full water system was the finest condition. Cruel comparison of genotypes based on primary spike weight shown that 'genotype 2' and 'genotype 7' had respectively the highest and lowest weight under full irrigation condition. The details are presented in Table 3. Also, 'genotype 7' had the lowest spike weight under stress conditions. Table 4 depicts the positive correlation between TGW, the number of grains per fundamental spike, and primary spike weight (0.01%).

Table 2. Analysis of variance results

S.O.V	df	Plant height	Main spike weight	1000 grain weight	Yield	Harvest index
Rep	3	397.26**	0.91	7.21	79.62	18.24
Condition	1	6458.26**	17.92**	501.26**	54.26	803.26**
Genotype	9	730.26**	2.01**	15.24*	224.26**	80.34**
C*G	9	280.19**	5.65**	13.24	217.25	57.25**
Error	57	101.26	1.54	33.28	413.25	8.65
CV	-	8.91	15.42	9.68	12.27	8.79

** (0.01) And * (0.05) significant levels

Table 3. Mean compression of traits

Genotypes	Treatments									
	PH		MSW		1000-W		Y		Hi	
	N	S	N	S	N	S	N	S	N	S
1	127	101	2.81	2.22	55.35	50.16	98.51	95.26	21.26	23.25
2	114	88	3.24	2.69	60.17	56.68	83.25	70.26	18.26	25.48
3	149	122	1.98	1.54	61.28	51.29	103.25	102.26	22.35	27.26
4	131	126	2.99	1.89	63.59	59.65	79.52	75.21	23.26	24.26
5	114	113	2.54	2.65	51.27	51.02	72.36	60.25	24.25	28.21
6	142	124	2.03	1.85	48.26	45.36	134.26	110.26	21.24	20.26
7	127	92	1.87	1.52	61.27	58.24	65.32	80.36	22.29	25.49
8	130	117	2.68	1.33	57.21	51.26	84.26	75.68	24.68	27.62
9	119	99	2.51	2.01	55.79	50.35	75.36	79.26	27.62	30.14
10	124	108	2.68	1.87	54.35	57.26	74.28	72.04	28.32	40.29

N: Normal, S: Stress, PH: Plant height, MSW: Main spike weight, 1000-w: 1000 grain weight, Y: Yield, Hi: Yield

TGW: As depicted in Table 2, the results of variance analysis indicated the following results concerning the significance of genotype impact for traits: critical in (0.05 percent level); inconsequential environment impact in 0.01 percent level and Genotype and environment interaction for TGW was non-Significant. The cruel comparison shown that full water system was the finest condition. The cruel comparison of genotypes based on TGW indicated that 'genotype 4' and 'genotype 6' had respectively the highest and lowest TGW in normal conditions. Also 'genotype 6' had the lowest TGW under stress condition, as depicted in Table 3. The results of correlation analysis are presented in Table 4, which reported the positive correlation between TGW and main spike weight.

Yield: As depicted in Table 2, the results of variance analysis indicated that the genotype had a significant impact on yield (0.01). Besides, the findings suggested that the impact of the environment and the interaction between genotype and the environment were non-significant. The cruel comparison demonstrated that full water system was the leading condition. The cruel comparison of genotypes based on abdicate appeared that 'genotype 6' had the highest yield and 'genotype 7' the lowest yield in normal condition. Also 'genotypes 5' represented the lowest yield under stress condition (Table 3). The findings of this study indicated a positive correlation between yield, total plant weight, TGW, and HI. Most researchers consider yield as a fundamental trait and put it at the focus of their research studies in the search for the optimal cultivar (Table 4).

Harvest index: The comes about of examination of change (Table 2) appeared that the impact of genotype, environment, and genotype and environment interaction for characteristics in terms of collect list was critical (0.01). The mean comparison indicated that the stress condition was the best condition. The mean comparison of genotypes based on HI showed that, under normal condition, 'genotype 10' had the highest and 'genotype 2' the lowest level of HI; Also, 'genotype 6' had the lowest HI value under stress condition, as represented by Table 3. As depicted in Table 4, the results of the correlation analysis indicated the positive correlation between HI and GY.

Table 4. Correlation between traits

	Plant height	Main spike weight	1000 grain weight	Harvest index	Yield
Plant height	1	0.42	0.048	-0.64**	-0.68
Main spike weight		1	0.412**	0.019	0.41
1000 grain weight			1	-0.078	0.27
Harvest index				1	0.801**
Yield					1

Table 5. Correlation coefficient analysis with direct and indirect effects for yield

Trait name	Direct effect	Indirect effects			
		Plant height	Main spike weight	1000grain weight	Harvest index
Plant height	0.17	1	-0.001	-0.001	-0.489
Main spike weight	0.031	0.006	1	-0.001	0.018
1000 grain weight	-0.003	0.009	-0.007	1	-0.051
Harvest index	0.804	-0.121	-0.002	0	1
Effects remain: 0.077					

Table 4. correlation between traits In similar studies evaluating methods' performance characteristics, the cause and effect model of interpretation was used to discuss and analyze the relationship between yield traits. This study used this model to analyze the correlation between yield and yield components' traits and identify the direct and indirect impacts of those traits. Table 5 presents the results of the path analysis. The result indicated that HI had the highest direct positive impact on GY while TGW had the highest negative impact. Also, most indirect effects by harvest index on plant height were applied.

Based on observations, day to complete heading was lower in tall plants, which meant a prolonged reproductive phase and more dense accumulation of photosynthates in the grains. Thus, the findings stated that plant height (PH) and grain yield (GY) were highly, significantly, and positively correlated. Similarly, the findings of relevant studies confirm such reports stating that GY was more considerable in taller plants as the number and size of grain in tall plants were higher. Plant height, on the other hand, though, had remarkable negative impacts on maturity days (Shamsuddin and Ali 1989), further confirming the findings of other studies (Subhashchandra, Lohithaswa et al. 2009).

Among all traits, the only trait with considerably negative perceived correlation with other traits was the 'days to maturity' trait. Other than, significant negative relationships were moreover watched for heading days with all the characteristics but development days, proposing a shorter vegetative stage together with a longer regenerative stage would contribute to higher grain surrender. Grain abdicate was emphatically related with plant tallness, the number of spikes/m2, and 1000-grain weight both at genotypic and phenotypic levels but critical as it were at the genotypic level. This result is also in line with the findings of Dogan (Dogan 2009) and Gashaw et al. (Gashaw, Mohammed et al. 2007).

They reported the correlation between GY and TGW was significant and positive. Besides, it was observed that GY and TGW did not have a significant genotypic and phenotypic correlation with days to heading and days to maturity, however, their correlations were remarkably high and negative. The findings of similar studies confirm the non-significant and negative association of GY and maturity days. (Subhashchandra, Lohithaswa et al. 2009) . It is stated that due to the prolonging of the vegetative phase, it wasn't to fixate photosynthesis translocate it to the developing grain, which significantly damaged GY and GS (grain size). The number of grains/spike negatively correlated with GY and TGW at the genotypic level. Their correlation wasn't significant, indicating that higher grain number negatively impacted grain size and in the end, damaged (decreased) grain yield.

CONCLUSION

The results indicated the relative resistance power of the genotypes studied in the experiment under drought stress conditions. Moreover, the observed high levels of TGW and main spike weight were indicators of their influence on the total yield. Therefore, this study states that investigating the impacts of such traits not only boosts the potential drought resistance of plants it will also improve yields. The findings and observations of this research study concerning the optimal genotypes combined with relevant data pave the way for other breeders and researchers in their attempts to find the best cultivars and improve yields in wheat durum wheat farms.

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In-Silico Sreening of T-cell Epitopes as Vaccine Candidate from Proteome of H9N2 Virus

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ABSTRACT

H9N2 virus outbreak has increased worldwide in last decade due to the zoonotic potential of these viruses. H9N2 virus causes low pathogenicity but when co-infected with other pathogen it causes high mortality. Since 1998, H9N2 infection has Caused one death and more than 59 cases as reported worldwide in animals including humans. There are currently no clear methods to control the pandemic potential of H9N2 virus globally, so there is urgent need for vaccine designing against these viruses.In this study, screening of T-Cell epitopes from H9N2 virus proteins viz nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 protein followed by highest binding affinity of selected T-cell epitopes with their corresponding HLA alleles has been done. The server ProPred1 & ProPred facilitates the binding prediction of HLA class I & class II allele with specific epitopes from the antigenic protein sequences of H9N2 virus. PEPstrMOD server was used structure modeling of the screened epitopes. We docked the selected T-cell epitopes with their corresponding HLA allele structures using the HPEPDOCK Server. Toxicity & immunogenicity of epitopes were analyzed by Toxin Pred and IEDB tools, respectively. The screened T-cell epitopes viz FQGRGVFEL, AEIEDLIFL, IIEGRDRTL, RRVDINPGH, YIGVKSLKL, LVMKRKRDS, VVLVMKRKR, LVRKTRFLP are anticipated to be valuable in designing comprehensive epitope-based vaccines against H9N2 virus after further *in-vivo* studies.

KEY WORDS: H9N2 VIRUS, T-CELL EPITOPE, HLA ALLELES, VACCINE DESIGNING.

INTRODUCTION

H9N2 viruses cause worldwide infections and the majority of confirmed cases are young children. Different combination of hemagglutinin and neuraminidase, surface proteins of Influenza A viruses, give rise to subtypes viz H1N1, H5N6, or H9N2. Different studies have showed that the primary routes of transmission of H9N2 virus are respiratory and direct contact. Aerosol, droplet particles, oral-facial route and direct touch are the other routes of transmission for this virus (Killingley et al., 2013). H9N2 virus infection has been observed in humans in Hong Kong, India, Bangladesh, Pakistan, Oman, Egypt and China (Butt et al., 2003; Shanmuganatham et al., 2013; Pan et al., 2018; Ali et al., 2019; Potdar et al., 2019). H9N2 outbreaks in commercial chickens from Asia, Middle East and African countries have also been reported recently (Li et al., 2020).

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A recent report of H9N2 virus (strain A/India/TCM2581/2019) infection was observed in a 17 month-old boy residing at Melghat District, Maharashtra, India , which showed a threat to human infection in India and there is an urgent need for the treatment of this emerging virus (Potdar et al., 2019). Important implication of this study is to screen promiscuous T-cell epitopes from H9N2 virus proteins viz nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 protein. The screened selected T-cell epitopes may be the promising targets for epitope-based vaccine design for H9N2 virus.

MATERIAL AND METHODS

Complete genome sequence of H9N2 virus strain (A/India/TCM2581/2019/(H9N2) was taken in this work (Potdar et al., 2019). The amino acid sequence of nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein and polymerase PB2 protein of H9N2 virus were retrieved from protein sequence database from NCBI (http://www.ncbi.nlm.nih.gov/protein) and their accession number were shown in Table 4. A proteomics server, ExPASy ProtParam (www.expasy.org) was used to analyze the primary structure of the target protein. Several parameters given by ProtParam tool for example estimated half-life, amino acid composition, theoretical pI, molecular weight, extinction coefficient, atomic composition, aliphatic index, grand average of hydropathicity (GRAVY) and instability index were examined. SOPMA (Geourjon and Deleage, 1995) server used to check the secondary structure (alpha helix, beta plated sheets, turns and coils) of the proteins, its aim to predict solvent accessibility, transmembrane helices, coiled-coil regions, globular regions and ultimately determines the stability and function of proteins.

To predict the protective antigens as vaccines, the sequence was then analyzed by VaxiJen.VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Doytchinova and Flower, 2007) with default parameters to find out the antigenicity. All the antigenic proteins with their respective predicted score were computed. The prediction of potential HLA class I & class II binding nanomer epitopes completed by using Propred I & Propred respectively (Singh and Raghava, 2001). Threshold percentage of highest scoring peptides is taken at 3%. Top four binders for different HLA allele have been taken into consideration. Immunoproteosome site and Proteasome site filters were put in 'on' mode with threshold of 4% for each filter.

ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) was used to predict toxicity of predicted T- cell epitopes (Gupta et al., 2013). ToxinPred is an in-silico tool to predict the selected epitope as toxic or non-toxic. ToxinPred was run with default parameters and only non-toxic T-cell epitopes were selected for further study.

The PEPstrMOD (Singh et al., 2015) method performed to find out the tertiary structure of selected nanomer epitopes. The PEPstrMOD tool prediction strategy utilizes the secondary structure data & β-turns data anticipated by PSIPRED and BetaTurns respectively. The amino acid sequences of HLA alleles were retrieved from IMGT/HLA database (http://www.ebi.ac.uk/ipd/imgt/hla/intro.html) (Robinson et al., 2012) and homology model of alleles was constructed using program HPEPDOCK (http://huanglab.phys.hust.edu.cn/hpepdock/) Server (Zhou et al., 2018).

HPEPDOCK Server has been used to perform docking of epitopes with alleles models. Docking studies was performed to study the interaction of epitopes with alleles. For such interaction studies, the most important requirement was the proper orientation and conformation of epitope, which fit to the binding site of the allele appropriately and form the epitope-allele complex. The obtained docking scores was tabulated and analysed. Immunogenicity of the selected T-cell epitopes was predicted by using IEDB (Immune Epitope Database and Analysis Resource) (http://tools.iedb.org/immunogenicity/) (Calis et al., 2013). This tool predicts the relative ability of an epitope-HLA complex to elicit an immune response. Amino acid properties & their position within the epitope are utilized by this tool to predict the immunogenicity of a class I epitope-HLA complex.

RESULTS AND DISCUSSION

H9N2 viruses are emerging zoonotic infectious viruses that cause fatal diseases in both animals and humans (Pusch and Suarez, 2018). New efficient vaccines against H9N2 virus infection are urgently needed to control the disease and its proliferation. In the present study, prediction and modeling of T cell epitopes of H9N2 virus antigenic proteins followed by docking studies of predicted highest binding scores with their corresponding HLA class I and class II alleles have been performed.

Primary and secondary structure analysis: Primary structure analysis viz theoretical isoelectric point (PI), molecular weight, total number of positively charged residues (Arg+Lys) and negatively charged residues (Asp+Glu), estimated half-life (*in vitro*) in mammalian reticulocytes and instability index (II) are shown in table 1 while secondary structure analysis viz alpha helix, extended strand, beta turn & random coil are shown in table 2.

Protein antigenicity determination: Amino acid sequences of proteins viz nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 were screened by VaxiJen. All the proteins were found antigenic except nuclear export protein & PB1-F2 protein which were non-antigenic at threshold value of 0.4 (default threshold for viral proteins) (Table 3). Antigenic proteins selected for further analysis.

Table 1. Primary structure analysis using ProtParam

Name of Protein	No. of amino acids	Molecular weight	Theoretical PI	Total no. of negatively charged residues (asp-Glu)	Total no. of positively charged residues (asp-lys)	Extinction coefficient	Estimated half-life	Instability index	Aliphatic index	Grand average of hydropathicity
Nonstructural protein 1	237	26966.89	5.51	36	31	34615	30	58.37	86.41	-0.354
matrix protein 2 matrix	97	11268.83	4.92	16	11	15595	30	57.86	92.37	-0.242
protein 1 Neuramini	252	27763.07	9.28	24	30	13075	30	36.32	85.99	-0.218
Dase Nucleo	469	51417.82	6.02	47	42	93610	30	33.69	75.22	-0.280
capsid Hemagg	498	56137.54	9.47	58	70	52745	30	44.25	70.76	-0.561
Lutinin	560	62616.94	6.91	56	55	87165	30	31.37	84.61	-0.345
polymerase PA	716	82568.39	5.54	112	95	95310	30	48.41	77.37	-0.469
polymerase PB2	759	85732.98	9.45	85	103	79090	30	45.89	87.80	-0.298

Table 2. The secondary structure analysis using SOPMA™

Protein	Alpha helix	Extended strand	Beta turn	Random coil
Nonstructural protein 1	132(55.70%)	30(12.66%)	7(2.95%)	68(28.69%)
matrix protein 2	49(50.52%)	14(14.43%)	4(4.12%)	30(30.93%)
matrix protein 1	153(60.71%)	24(9.52%)	16(6.35%)	59(23.41%)
Neuraminidase	32(6.82%)	162(34.54%)	29(6.18%)	246(52.45%)
Nucleocapsid protein	218(43.78%)	60(12.05%)	31(6.22%)	189(37.95%)
Hemagglutinin	192(34.29%)	117(20.89%)	42(7.50%)	209(37.32%)
polymerase PA	388(54.19%)	79(11.03%)	30(4.19%)	219(30.59%)
polymerase PB2	289(38.08%)	147(19.37%)	44(5.80%)	279(36.76%)

Prediction and analysis of HLA Class I & Class II binding peptides: H9N2 virus proteins were subjected to Propred1 & Propred for selection of HLA Class I & HLA Class II specific T- cell epitopes binders respectively. Epitopes showing highest score with the maximum number of HLA alleles binders were selected at a threshold value of 3% (Table 4).

Toxicity prediction: ToxinPred (Gupta et al., 2013) used for toxicity prediction of selected T- cell epitopes. ToxinPred tool is a unique in-silico method based on Support Vector Machine (SVM) in predicting toxicity of peptides along with important physico-chemical properties viz Charge, Hydrophobicity, Hydropathicity, Hydrophilicity and Molecular weight. The selected epitopes were subjected to ToxinPred and only non-toxic T-cell epitopes were selected for further studies (Table 5).

Molecular Docking: 3D structures of selected epitopes were predicted by PEPstrMOD while HPEPDOCK Server was employed to generate homology model of alleles. Template PDB ID (protein data bank) formed by server was used for alleles model (table 6). HPEPDOCK Server has been utilized to perform docking study of epitopes with alleles models (Figure 1-8). The best conformation of docked complex was chosen on the basis of minimum docking score (table 7).

Table 3. VaxiJen result of antigenicity

S.No.	Protein	Overall Antigen Prediction
1	nuclear export protein	0.3441 (Probable NON-ANTIGEN)
2	nonstructural protein 1	0.4290 (Probable ANTIGEN)
3	matrix protein 2	0.5641 (Probable ANTIGEN).
4	matrix protein 1	0.4805 (Probable ANTIGEN)
5	Neuraminidase	0.5513 (Probable ANTIGEN)
6	nucleocapsid protein	0.5208 (Probable ANTIGEN)
7	Hemagglutinin	0.4322 (Probable ANTIGEN)
8	polymerase PA	0.5273 (Probable ANTIGEN)
9	PB1-F2 protein	0.1654 (Probable NON-ANTIGEN)
10	polymerase PB2	0.5291 (Probable ANTIGEN).

Table 4. ProPred1 & ProPred predicted T-cell epitopes for HLA Class I & Class II with binding scores

Protein name	Amino acid length	Accession no.	Position	Epitopes	HLA class alleles	Propred (% of highest score)
nucleocapsid protein	498	QBP33428.1	457-465	FQGRGVFEL	HLA-B*0705	3000.000
nucleocapsid protein	498	QBP33428.1	250-258	AEIEDLIFL	HLA-B*2705	3000.000
polymerase PA	716	QBP33426.1	77-85	IIEGRDRTL	HLA-B*5101	387.200
polymerase PB2	759	QBP33423.1	142-150	RRVDINPGH	HLA-B*2705	9000.000
hemagglutinin	560	QBP33427.1	316-324	YIGVKSLKL	DRB1-0703	72.41
polymerase PB2	759	QBP33423.1	732-740	LVMKRKRDS	DRB1-1301	89.77
polymerase PB2	759	QBP33423.1	730-738	VVLVMKRKR	DRB1-1328	59.09
polymerase PB2	759	QBP33423.1	210-218	LVRKTRFLP	DRB1-1327	55.68

Table 5. Toxicity prediction of the peptides by ToxinPred

PEPTIDE SEQUENCE	SVM SCORE	PREDICTION	HYDROPHOBICITY	HYDROPATHICITY	HYDROPHILICITY	CHARGE	MOL WT
FQGRGVFEL	-1.37	NON-TOXIN	-0.05	0.14	-0.23	0.00	1052.33
AEIEDLIFL	-0.94	NON-TOXIN	0.16	1.19	-0.13	-3.00	1062.36
IIEGRDRTL	-0.94	NON-TOXIN	-0.32	-0.48	0.69	0.00	1072.36
RRVDINPGH	-0.91	NON-TOXIN	-0.44	-0.39	0.60	1.50	1063.31
YIGVKSLKL	-1.14	NON-TOXIN	0.01	0.67	-0.32	2.00	1020.42
LVMKRKRDS	-0.84	NON-TOXIN	-0.60	-1.24	1.19	3.00	1132.51
VVLVMKRKR	-0.71	NON-TOXIN	-0.37	0.17	0.49	4.00	1128.62
LVRKTRFLP	-0.65	NON-TOXIN	-0.30	-0.07	0.11	3.00	1129.54

Docking of selected nanomer T-cell epitopes FQGRGVFEL, AEIEDLIFL, IIEGRDRTL, RRVDINPGH, YIGVKSLKL, LVMKRKRDS, VVLVMKRKR, LVRKTRFLP with their corresponding allele HLA-B*0705, HLA-B*2705, HLA-B*5101, HLA-B*2705, DRB1-0703, DRB1-1301, DRB1-1328, DRB1-1327 respectively showed stable HLA–peptide complexes with docking score -229.708, -184.637, -206.640, -197.206, -176.581, -159.444, -177.159, -227.036 respectively (Table 7).

Epitope antigenicity determination: VaxiJen is used with default parameters to predict the antigenicity of epitopes

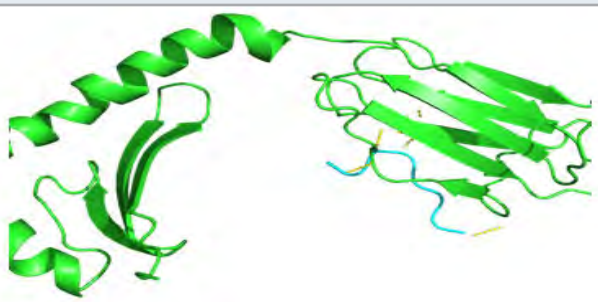
as vaccines candidate. All the antigenic epitopes with their respective predicted score (value greater than 01) were selected (table 8 & 9).

Table 6. Template PDB ID for modeling of selected HLA alleles

S.No.	Allele	Template of model	Sequence Identity
1	HLA-B*2705	6AT5 A	92.8%
2	DRB1*1328	6ATF B	92.1%
3	HLA-B*0705	6AT5 A	99.4%
4	HLA-B*5101	6AT5 A	90.9%,
5	DRB1*0703	4H25 B	84.3%
6	DRB1*1301	6PX6 B	66.3%
7	DRB1*1327	6PX6 B	66.3%,

Figure 1: Docked complex of Nucleocapsid protein epitope AEIEDLIFL& HLA-B*2705 allele

Figure 3: Docked complex of polymerase PAprotein epitope IIEGRDRTL & HLA-B*5101allele



Immunogenicity prediction of selected epitopes: Immunogenicity of nanomer HLA class I selected epitopes were analysed by IEDB. The selected epitopes with positive value showed high immunogenicity (Table 10).

The selected T-cell epitopes FQGRGVFEL, AEIEDLIFL, IIEGRDRTL, RRVDINPGH, YIGVKSLKL, LVMKRKRDS,

Figure 2: Docked complex of Nucleocapsid protein epitope FQGRGVFEL& HLA-B*0705allele

Figure 4: Docked complex of polymerase PB2 protein epitope LVMKRKRDS & DRB1-1301 allele

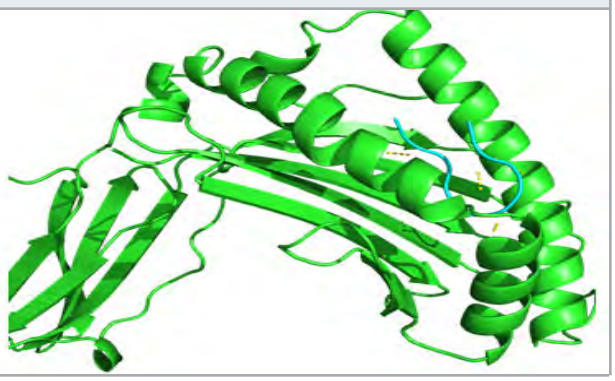


Figure 5: Docked complex of polymerase PB2 protein epitope LVRKTRFLP & DRB1-1327 allele

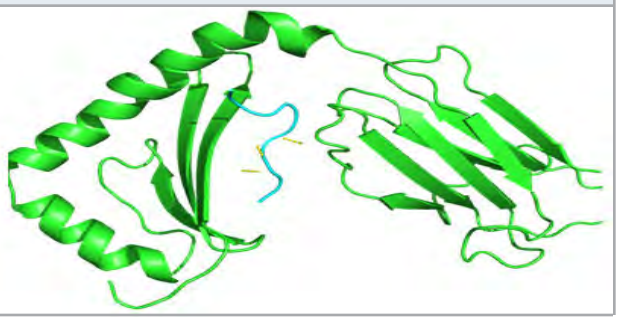
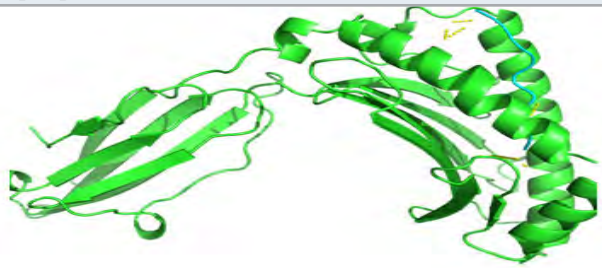


Figure 6: Docked complex of polymerase PB2 protein epitope RRVDINPGH & HLA-B*2705 allele



VVLVMKRKR, LVRKTRFLP also show positive values of antigenicity & immunogenicity (in case of HLA class I) as shown in table 8-10. We have previously published similar work for HLA class I alleles for Nipah and HLA class II alleles for Hendra viruses (Kamthania and Sharma, 2015; Kamthania et al., 2019).

Figure 7: Docked complex of polymerase PB2 protein epitope VVLVMKRKR &tDRB1-1328 allele

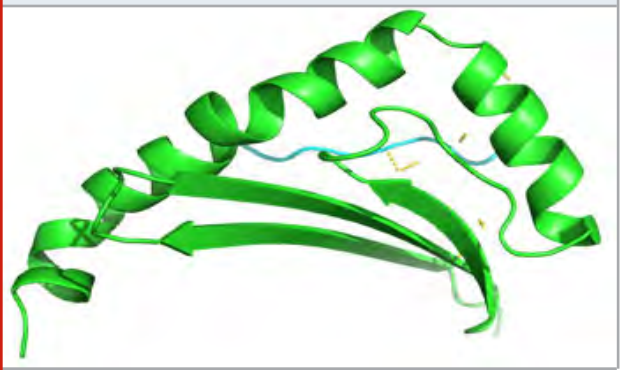


Figure 8: Docked complex of hemagglutinin proteinepitope epitope YIGVKSLKL&tDRB1-0703 allele

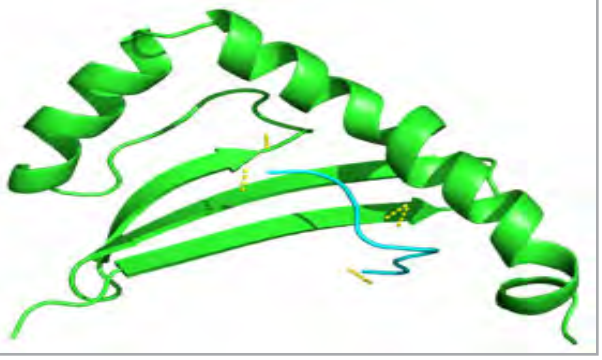


Table 7. Docking result of selected T-cell epitopes with allele structures.

S. No.	Protein name	Epitopes	HLA class alleles	Docking Score
1	nucleocapsid protein	FQGRGVFEL	HLA-B*0705	-229.708
2	nucleocapsid protein	AEIEDLIFL	HLA-B*2705	-184.637
3	polymerase PA	IIEGRDRTL	HLA-B*5101	-206.640
4	polymerase PB2	RRVDINPGH	HLA-B*2705	-197.206
5	hemagglutinin	YIGVKSLKL	DRB1-0703	-176.581
6	polymerase PB2	LVMKRKRDS	DRB1-1301	-159.444
7	polymerase PB2	VVLVMKRKR	DRB1-1328	-177.159
8	polymerase PB2	LVRKTRFLP	DRB1-1327	-227.036

Table 8. Vaxijen for HLA class-I epitopes

S. No.	SEQUENCE	VAXIJEN RESULT
1	FQGRGVFEL	1.2783 (PROBABLE ANTIGEN)
2	AEIEDLIFL	1.0317 (PROBABLE ANTIGEN)
3	IIEGRDRTL	1.2600 (PROBABLE ANTIGEN)
4	RRVDINPGH	2.5888 (PROBABLE ANTIGEN)

Table 9. Vaxijen for HLA class-II epitopes

S. No.	SEQUENCE	VEXIJEN RESULT
1	YIGVKSLKL	1.9299 (PROBABLE ANTIGEN)
2	LVMKRKRDS	1.9837 (PROBABLE ANTIGEN)
3	VVLVMKRKR	2.4990 (PROBABLE ANTIGEN)
4	LVRKTRFLP	1.6827 (PROBABLE ANTIGEN)

Table 10. Immunogenicity of HLA class I epitopes

S.NO	SEQUENCE	EPITOPE LENGTH	IMMUNOGENIGITY SCORE
1	FQGRGVFEL	9	0.29224
2	AEIEDLIFL	9	0.33583
3	IIEGRDRTL	9	0.20424
4	RRVDINPGH	9	0.16967

CONCLUSION

In this study, we have identified the potential nanomer T-Cell epitopes as vaccine candidate against H9N2 virus. The results confirming high binding affinity of selected epitopes with HLA alleles, stable complex formation tendency with HLA allele and tendency to induce high and specific immunogenic response makes the selected nanomer T-Cell epitopes to be a potential candidate for epitope based vaccine development against H9N2 virus

infection. Hence reported nanomer epitopes may undergo further in-vivo trials to develop vaccine against H9N2 virus infection.

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Evaluation of Optic Disc Area Using Cirrus HD- Optical Coherence Tomography in Saudi Population

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ABSTRACT

The present study was carried out to determine the normative value of the optic disc size, interocular and gender differences and correlate disc size to glaucoma patients measured with Cirrus HD-OCT (Carl Zeiss Meditec, Inc., Dublin, California) device in Saudi population. Retrospective cross-sectional study which included 416 subjects was done. Optic nerve head imaging was obtained by Cirrus HD-OCT (Carl Zeiss Meditec, Inc., Dublin, California) using optic disc scans. Cirrus HD-OCT with the optic disc cube 200x200 protocol was implemented for all subjects, which was divided into 3 groups: normal, glaucoma suspects and glaucoma patients and comparison between groups was carried out using ANOVA test. The mean age of subjects included in the study was 54.9 years ± 15.38 with 47.8% males and 52.2 % females. Comparison of the interocular difference in disc area in the same subject was found to be slightly larger in the right eye than the left eye (1.98 mm and 1.97 respectively) (ns). Disc area in females was significantly larger than males (p-value= 0.042). Glaucoma suspect patients had larger disc area with an average of 2.19 mm2 in comparison to normal and glaucoma patients (p-value= 0.004). Cirrus HD-OCT is essential for optic disc analysis and quantitative assessment of the ONH. The observed differences in ONH measurements between females and males, glaucoma suspect, and other groups needs further longitudinal studies to evaluate its clinical importance and glaucoma associated risk. In addition, further studies comparing disc size in different regions of Saudi Arabia are recommended.

KEY WORDS: GLAUCOMA; MEASUREMENT TECHNIQUES; OPTIC DISK AREA; OPTICAL COHERENCE TOMOGRAPHY.

INTRODUCTION

The optic disc represents the start of the optic nerve and it is the point where the axons of retinal ganglion cells come together and exit the eye globe. The optic disc is located 3 to 4 mm from the nasal side of the fovea. It is a vertical oval, with average dimensions of 1.76mm horizontally by 1.92mm vertically (Duane TD, 2006).Direct measurement of the optic disc size is only possible during vitreoretinal surgery or histologically(Garway-Heath et al., 1998). Therefore, several correction factors have been developed to compensate for the camera and eye magnification error (Ansari-Shahrezaei and Stur, 2002). The optical coherence tomography (OCT) has been used to assess optic disc topography in clinical practice. This device provides in vivo cross-sectional scans of retinal structures by the use of low-coherence interferometry (Huang et al., 1991) (Wang et al., 2019).

Ophthalmological assessment of the optic disc is essential for the ocular and neurological health (Hoffmann et al., 2007) (Jonas and Budde, 2000). In particular, the assessment of glaucoma diagnosis and evaluation of progression (Jonas et al., 1999) (Michelessi et al., 2015). yet, it is debatable whether disc size is an independent risk factor for glaucoma or not (Hoffmann et al., 2007). Physiologically, disc size is known to vary largely between populations, among individuals and between

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eyes (Mansour, 1991) (Bass and Sherman, 2004) (Mays El-Dairi, 2020). The Baltimore Eye Survey report showed significantly larger discs in males compared to females (Varma et al., 1994). Moreover, African Americans found to have a larger discs compared to white individuals (Varma et al., 1994) (Ocansey et al., 2020). In regard to age, it does not appear to be associated with disc size in humans. In (Jonas et al., 1991) study , optic disc size in patients with glaucoma was slightly larger than in healthy individuals (Bayer et al.2020).

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These differences must be considered when evaluating the optic disc. Every clinician has at one time or another examined a patient who was misdiagnosed as glaucoma or whose diagnosis of glaucoma was missed. Although glaucoma can exist with normal intraocular pressure, clinicians often rely on the presence of visual-field defects, the degree of optic disc cupping and retinal nerve fiber layer thickness measurement by OCT (Girkin et al., 2003) (Tao et al., 2017) (Lee et al., 2018) (Fox 2020). Up to the present knowledge, there are no studies conducted in Saudi Arabia to evaluate the optic disc size and correlate the disc size to glaucoma susceptibility. The aim of the current study was to determine the optic disc size, interocular and gender differences and correlate disc size to glaucoma patients measured with Cirrus HD-OCT (Carl Zeiss Meditec, Inc., Dublin, California) device.

MATERIAL AND METHODS

The study is a retrospective cross-sectional study carried at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The study adhered to the tenets of the Declaration of Helsinki. Medical records from glaucoma clinic was reviewed from January 2019 to December 2019. The data included were the patient age, gender, glaucoma status, intra-ocular pressure. Optic disc size measured using Cirrus HD-OCT (Carl Zeiss Meditec, Inc., Dublin, California) optic disc scans. Cirrus HD-OCT with the optic disc cube 200x200 protocol was implemented for all patients. Subjects were divided into 3 groups as shown in table 1. Inclusion criteria were age >18 years, good OCT scan quality defined as scans with signal strength ≥ 6. Exclusion criteria were poor OCT scan quality with signal strength <6, optic nerve disc pathology such as papilledema and congenital optic nerve anomaly.

Table 1. Subjects groups characteristics

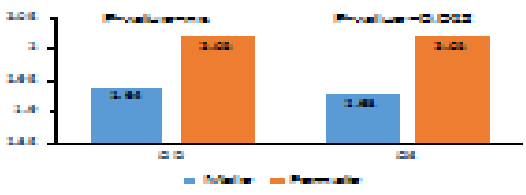
Group	IOP	Optic Disc	Visual field
Normal	≤ 21mmHg	a. C/D* ratio ≤ 0.5 b. no asymmetric C/D ratio ≥0.2 c. absent of notching or narrowing of the neuroretinal rim	Normal vf
Glaucoma suspect[1]	>21mmHg	a. C/D > 0.5 b. asymmetric C/D ratio ≥0.2 c. notching or narrowing of the neuroretinal rim	Visual field abnormality consistent with glaucoma
Glaucoma[2]	>21mmHg	a. C/D > 0.5 b. asymmetric C/D ratio ≥0.2 c. notching or narrowing of the neuroretinal rim	Glaucomatous visual field defect

*C/D: cup to disc ratio
1 Prescence of at least 1 of the finding
2 Presence of at least 2 findings

Table 2. average disc area in group subjects

Glaucoma classification	Frequency	Avg. Disc area	P value
Normal	152 (36.6 %)	1.92 mm	0.004
Glaucoma suspect	109 (26.2 %)	2.19 mm	
Glaucoma	155 (37.2%)	1.95 mm	

Figure 1: Comparison of disc area between males and females



Statistical Analysis: Data were analyzed using IBM SPSS software (version 23). Descriptive analysis of variables (frequency, mean and standard deviation) was performed. Independent t-test was used for gender comparison in regard optic disc area. When comparing right eye disc area and left eye disc area, dependent t-test was used. Univariate analysis, which compared differences between the glaucoma, glaucoma suspects and normal groups, was done using ANOVA. A P-value of < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Total of 416 subjects were included in the study (47.8% male and 52.2 % female) with average age of 54.9 years ± 15.38. When comparing disc area for both eyes in the same subject, Right eye (OD) found to be slightly larger disc area compared to the left eye (OS) (1.98 mm and 1.97 respectively), ns. Differences between male and female disc area are shown in Figure 1. In regards of glaucoma classification group, disc area measurement is shown in table 2.

Several techniques such as planimetric, biomicroscopic, confocal scanning laser ophthalmoscopy (CSLO) and optical coherence tomograph (OCT) have been used to measure disc area in which all are generally strongly correlated (Hoffmann et al., 2007) (Wang et al., 2019). (Ruben, 1994) found resemblance in disc size between planimetry and biomicroscopic disc size measurements. Moreover, (Garway-Heath et al., 1997) comparison between planimetric and CSLO measurements of disc size showed no difference in normal subjects. Several reports showed correlation between OCT topographic optic disc measurements and CSLO measurements (Hoffmann et al., 2005) (Medeiros et al., 2005) (Schuman et al., 2003) . However, several reports showed that CSLO measurements of disc area is likely to be smaller than that of the OCT (Schuman et al., 2003) (Fox 2020).

The size of the optic disc can varies according to race and African American found to have larger disc area in comparison to other races (Mansour, 1991) (Varma et al., 1994) (Zangwill et al., 2004) (Ocansey et al., 2020) . Up to knowledge, no previous reports have estimated disc area in Saudi population. The mean disc in the studied subjects was 1.9mm2 ±0.45mm2 which is smaller than African American and correlated to other ethnic groups (Zangwill et al., 2004) (Ocansey et al., 2020).

It was hypothesized that Africans and African American are more susceptible to develop glaucomatous disc damage due to a larger disc size (Burk et al., 1992). However, several reports explained that different factors could explain the higher frequencies of glaucoma in African-Americans such as genetic predisposition to primary open angle glaucoma (POAG), higher chronic diseases (cardiovascular disease, diabetes and systemic hypertension), and thin corneal thickness (Fingert et al., 1999) (Herndon et al., 2004) (Grzybowski et al., 2020).

In the literature, studies have assessed the optic disc area differences based on gender (Varma et al., 1994) (Zangwill et al., 2004). (Bowd et al., 2002) revealed no difference in disc area between males and females using CSLO. However, a large population based survey showed significantly lager disc area in males compared to females (Varma et al., 1994) (Quigley et al., 1999) (Bayer and Akman, 2020). In contrary, this study demonstrated larger disc area in females compared to males.

Correlation between disc size and glaucoma susceptibility have been evaluated in several studies (Jonas et al., 2004) (Iester et al., 1997) (Healey and Mitchell, 1999) (Mittal et al., 2018). Comparison of optic disc area between healthy people, ocular hypertension, POAG and secondary glaucoma patients using planimetric and CSLO found no difference in disc area among them (Iester et al., 1997) (Jonas et al., 2004) . However, the Blue Mountains Eye Study revealed a minimal difference in disc size in which ocular hypertensive eyes had smaller discs than patients with primary open angle glaucoma (Healey and Mitchell, 1999). Previous longitudinal studies were conducted to assess whether optic disc area is an associated risk factor for glaucoma development or progression or conversion from ocular hypertension to glaucoma (Jonas et al., 2004) (Zangwill et al., 2005) (Wang et al., 2019) . These reports showed that optic disc area was not significantly neither associated with risk of conversion nor progression (Jonas et al., 2004) (Zangwill et al., 2005) (Wang et al., 2019) (Grzybowski et al., 2020).

This study showed difference in disc area between normal subjects, glaucoma suspects and glaucoma patients in which glaucoma suspect showed larger disc area compared to other groups. However, large disc area is associated with increase the optic disc cup which can be misdiagnosed as glaucomatous cupping. The main limitation of the study is that it was a retrospective single-center study. A multicenter study should be conducted to evaluate the disc area measurement over different regions of the country and estimate normative reference value in our population. Up to knowledge, this is the first study conducted to evaluate disc area measurement in healthy and glaucoma patients using cirrus optical coherence tomography in Saudi population.

CONCLUSION

Evaluation of optic disc size is an important part of ophthalmic examination specially in glaucoma diagnosis and management. Additionally, Cirrus HD-OCT is a valuable tool for disc analysis in clinical practice and for long-term follow up. This is in conjugation with other methods for disc assessment will enhance patients care in ophthalmology practice. Longitudinal studies are required to evaluate the correlation between disc area and other risk factors for glaucoma development such as intraocular pressure, central corneal thickness and high refractive errors using OCT and analyze its correlation with other diagnostic methods.

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Biogenic Platinum as Nanomedicine: A Synergism of Ethnomedicine and Nanotechnology

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ABSTRACT

This rationale of the present work is to amalgamate the benefits of ethnomedicine and nanotechnology by fabricating biogenic platinum nanoparticles to look for possible alternative treatment to cure fatal diseases like cancer. Biogenic Platinum nanoparticles were fabricated from the leaves of medicinal plant of *Piper betle*. The fabrication was ascertained in the umbrella of tools that helped in its characterization and included spectroscopic techniques like UV-vis and FTIR along with X-Ray diffraction and Scanning electron microscopy. The cytotoxicity assay against Lung cancer cell line (A549) was carried out by the MTT assay technique to check the cytotoxic efficacy of Platinum nanoparticles in comparison to that of the ethnomedicinal *Piper betle* plant extract. It was affirmed that both PtNPs and plant extract reduces the cell viability of cancer cells but at all the doses PtNPs proved to be more efficient in their cytotoxic effect in comparison to the extract. This work is a small step towards foreshadowing possible alternative therapeutics for cancer and revolutioninzing our ancient ethnomedicine.

KEY WORDS: ETHNOMEDICINE, NANOTECHNOLOGY, PLATINUM NANOPARTICLES, PIPER BETLE, LUNG CANCER CELL LINE (A549).

INTRODUCTION

Ethnomedicine or traditional herbal medicine is an ancient science that is known for curing disease using indigenous plants. Plants as therapeutic agents can be used in various ways like isolating bioactive compounds for direct use as drugs or directly using the whole plant/part of it as an herbal remedy. Toxicity concerns with use of plants are less likely as compared to chemicals. Each medicinal plant is blessed with an array of phytochemicals like alkaloids, flavonoids, terpenes, tannins, organic acids etc. that play important roles in several biological activities, including antibacterial, antifungal, anti-inflammatory, antitumour etc. (Madhumita et al., 2020).

This treasure of diverse organic compounds in these plants is also known to play an important role in nanotransformation through molecular level modifications. Thus, making plants a suitable candidate for nanoparticle synthesis. On the other hand, the path-breaking, scientifically fascinating branch of nanoscience and nanotechnology has witnessed a revolution since its genesis in the exploration of matter at reduced dimensions. The special functionality flaunted by nanosized matter has been recognized leading to their wide applications in various sectors; biomedical being one of them. Platinum nanoparticles are one of the eminent nanoparticles with immense usage and practical applications in the

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biomedical sector that allured scientists to search out various fabrication routes for their synthesis (Jha et al., 2018a).

This work is an attempt to synergize the ethnomedicine and nanotechnology streams by fabricating biogenic platinum nanoparticles from the time-honored plant, *Piper betel* well known for its medicinal values. The cytotoxic potential of the rich phytoconstituents of the leaves against the cancer cell line is a proven fact (Abdul Rahman et al., 2014, Gundala et al., 2014, Ng et al., 2014). Although the effect of bare platinum as a cytotoxic agent is reported in chunks platinum as conjugates have been experimentally proven to be effective against a wide range of cancer cells, (Porcel et al 2010, Oberai et al 2014, Cheng et al., 2016, Johnstone et al., 2016, Jha et al 2018b, Madhumita, et al. "2020".

This work aims to amalgamate the benefits of ethnomedicine and nanotechnology by fabricating platinum from the medicinal leaves extract. The active metabolites besides helping in fabricating the platinum also bind with the nanoparticles and stabilize thereby reducing their toxicity. This adds on therapeutic advantage to the synthesized nanoparticles and also drastically reduces the side effect. This effort/approach escorts the development of alternative therapeutic treatment for fatal diseases like cancer.

MATERIAL AND METHODS

Fresh and healthy leaves of *Piper betle* plant were procured and thoroughly washed initially with tap water and then after with distilled water. In order to disinfect it was further sterilized with ethanol and left for some time later washed with sterile water and finally air dried. 10 gm of this air dried leaves was measured and finally chopped maintaining sterile environment. The chopped leaves were put in a beaker with 100 ml of 50% ethanol and placed on a boiling steam bath for 15 minutes till the solution turned green. When cooled the solution was double filtered to be used as source extract for aiding nanotransformation. 0.025 M Platinum salt solution in distilled water using analytical grade Platinum (IV) chloride salt from Hi-media Lab Pvt. Ltd., Mumbai, India was prepared by dissolving appropriate weight. 20ml of plant extract salt solution was diluted by 80 ml of distilled water and was placed on boiling water bath.

10ml Pt salt solution was added maintaining the basic pH conditions through the salt solution of analytical grade sodium hydrogen carbonate (Hi-media Lab Pvt. Ltd., Mumbai). The plant aided nanotransformation to obtain PtNPs was allowed to happen at the temperature of boiling water bath. As the reaction proceeded the nanoparticles formed in the duration of 40-45 min as a colloidal solution which on cooling was centrifuged at 10,000 rpm for 20 minutes. The pellet obtained after discarding the clear supernatant was washed two times with distilled water. After removal of moisture content, it was characterized before carrying out further investigation. The ethanolic extract of the medicinal plant was also retained to check its medicinal values in comparison to PtNPs.

The fabrication of PtNPs was ascertained by characterization techniques. These techniques help to congregate information needed for the preferment of fabrication techniques and further property based applicability of nanoparticles. The biosynthesized PtNPs were characterized to know about their properties, dimensions, morphology etc. through various sophisticated instruments like UV-Vis spectroscopy, FTIR spectroscopy, X-Ray diffraction and Scanning electron microscopy. Perkin Elmer spectrophotometer, UK was used to record the UV-vis. Absorbance spectrum of the nanoparticle solution. The degree of precursor Pt metal ions conversion to their respective PtNPs was assessed by UV-Vis spectra (Behzadi et al., 2015, Hartland, 2006).

The FTIR spectrum of the dried PtNPs was collected in transmission mode in between the wavelength range of 4000-400 cm-1 by Perkin Elmer, UK, FTIR spectrophotometer. Bruker D8-Advance diffractometer with Cu-Kα radiation source aided in recording the X-Ray diffraction pattern of fabricated PtNPs (Bunaciu et al., 2015). It was used to determine the crystal structure and average particulate size. The SEM micrograph recorded by the EVO 18, Carl Zeiss Microscopy Ltd., helped in exploring the microstructural studies of biogenic PtNPs. The imaging provides topographical information about the nanoparticles.

It is of pivotal interest to assess the cytotoxicity of promising compounds intended for pharmaceutical use. The cytotoxicity of PtNPs was monitored by metabolic and imaging assays. MTT assay or metabolic assay provides information about the alterations in metabolism of cells in response to toxicities by promising compounds (Berridge et al., 2005) while imaging assays are employed to actuate the cytotoxic changes due to treatment. Lung cancer cell line (A549) were undertaken for the comparison of cytotoxic assay of medicinal plant extract and PtNPs (Gazdar et al., 2010). The cell line was cultured in RPMI 1640 media with 10% FBS and antibiotic pen-strep, all obtained from Hi Media Mumbai, India. The cells were placed in two 96 well plate with each well containing $5x10^3$cells in 200µl per well.

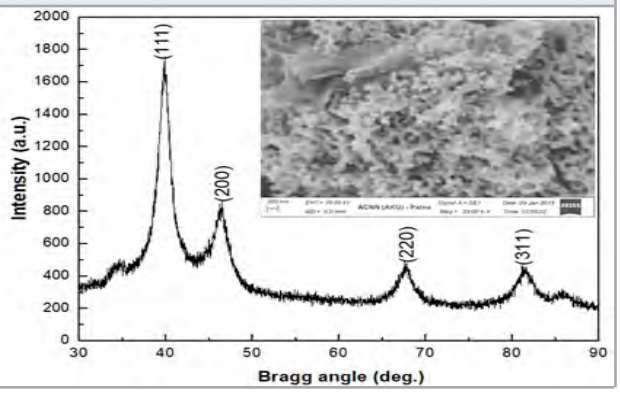
For the growth of mammalian cells physiological pH and temperature was maintained for 24 hr at 37°C in a 5% CO_2.The other day, the cells were treated with various concentrations of 5, 10, 25, 50, 75, 100, 150 and 200µg/ml of plant extract and PtNPs respectively. After a treatment period of 24hrs the media was aspirated and 100µL of MTT (Hi Media Mumbai, India) of concentration 5mg/ml in 1X PBS was appended to each well. Negative control for the experiment was a well with media containing no drug. After 4hrs of incubation in an incubator the MTT was removed from the 96 well plates and 100µL of DMSO was added per well. The readings were taken at 570nm and cell viability was represented as percent cell viability. The experiment was executed in triplicates for statistical analysis and reliability of results.

RESULTS AND DISCUSSION

Biogenic PtNPs fabrication: The medicinal plant extract of *Piper betle* served as a cocktail of multifunctional reactants acting both as reducing and stabilizing agents (Alam et al., 2013). In the present context the ethanolic extract of *Piper betle* with ethnomedicinal properties is store house of rich phytochemicals with abundant alkaloids, terpenoids, flavonoids and steroids that aid up in nanotransformation (Punuri et al., 2012). The metabolites act as reducing agents providing the electron to metal ion for nanotransformation. The reduction potential of the metal ion and the reducing metabolites both determines the effectiveness of the concerned bio-generators. With the development of nucleation centre by formation of elemental atoms, the nucleation process begins and the particle growth stages follows (Thanh et al., 2014).

The diminishing size results in high surface energy leading to aggregation of nanoparticles. The plant extract serving as a capping agent comes into action by surrounding the nanoparticles with biocompatible metabolites (Alam et al., 2013). The preliminary confirmation of this transformation was the visual inspection. On proper maintenance of processing parameters, the plant metabolites acting both as reducing and capping agents acts on salt solution to produce initial Pt nuclei that later undergo subsequent growth to give rise to stable, homogenous and capped PtNPs. The mixing of the precursor salt to the extract resulted in gradual colour change that after a duration of 40-45 minutes turned black confirming the completion of the reaction, (Attard et al., 2012, Venu et al., 2011 Jha et al 2018b).

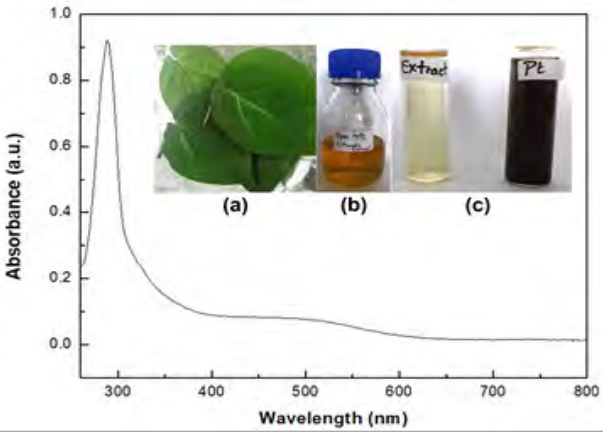
Figure 1: X-ray diffraction pattern and scanning electron microscope image, 3300 KX (inset) of PtNPs synthesized from the ethanolic extract of *Piper betle* leaves.



Structural and Microstructural Analysis of PtNPs: The further appliance of biogenic platinum nano entities is dependent on monitoring of its fabrication and properties which was achieved through characterization tools. Elucidation of PtNPs has been resoluted by numerous characterization techniques. The crystalline nature of biogenic PtNPs was established by the X-ray diffraction analysis. The Bragg reflections were observed as peaks in the graph plotted by Origin 8.5 data analysis and graphing software with the intensity on Y-axis and two times of Bragg angle (2θ) on the X-axis. As depicted in Figure 1, four peaks or reflection in the pattern was obtained each due to diffracted X-rays from a specific set of planes in the crystalline structure (Bunaciu et al., 2015).

Miller indices were assigned to the peak as (111), (200), (220), (311) and (222) which depicted face-centered cubic (fcc) structure. Applying Debye-Scherrer formula: $D = 0.94\lambda/\beta\cos\theta$ (where β = full width at half maxima of the peak) the apparent crystallite size was estimated to be ~7nm which was in accord with standard literature (ICDD no. #88-2343). The lattice parameter and volume of the cubic crystal structured PtNPs was estimated to be 3.9120Å and 59.868Å^3 respectively and were compared to the standard crystallographic bulk data to assess the squeezing in dimensions. The lowering in unit cell volume as observed could be attributed to nanosizing effect or quantum sizing effect (Jha and Prasad, 2010). The lattice strain value of PtNPs as observed was ~0.511 which owes to advantage of biosynthetic protocol. Upon interaction of the beam of electrons with the PtNPs the scanned surface of the nanoparticles was observed in the form of SEM micrograph as depicted by Figure 1, inset. The surface topography of PtNPs turned out to be nearly spherical structures.

Figure 2: UV-Visible spectrum of PtNPs synthesized from the ethanolic extract of *Piper betle* leaves. Inset shows the (a) *Piper betle* leaves, (b) ethanolic extract of *Piper betle* leaves and (c) diluted ethanolic extract of *Piper betle* leaves and PtNPs in colloid form.



UV-Vis. and FTIR spectra of PtNPs: The UV-Vis spectrum of the PtNPs fabricated by *Piper betle* leaves is shown in Figure 2. Besides, insets of Figure 2 depict (a) the *Piper betle* leaves, (b) ethanolic extract of *Piper betle* leaves and (c) diluted ethanolic extract of *Piper betle* leaves and PtNPs colloid. This spectrum studies comprehend the information for the final formation of nanoparticles. The colour generation of PtNPs (inset, Figure 2c) owe to the excitation of surface plasmon resonance caused on interaction with candidate metabolites. The λmax or the

wavelength of maximum absorbance was centralized at 288nm for PtNPs which ascertained the characteristic absorbance of platinum nanoparticles.

The spectrum showed long tail towards the longer wavelength that could be due to greater size distribution in these particles. FTIR spectroscopy is a label-free technique that enables extraction of biochemical information of the material under consideration. In the present work the fabricated PtNPs when subjected to infrared radiation results in a spectrum as presented in Figure 3. The metabolites aiding in nanotransformation tend to adhere to the fabricated PtNPs and the varied bonds of the metabolite absorbs varied intensities resulting in spectral domains. Each spectral peak is directly correlated to the bonds leading to deciphering and chemical identification.

Figure 3: FTIR spectrum of PtNPs synthesized from the ethanolic extract of *Piper betle* leaves.

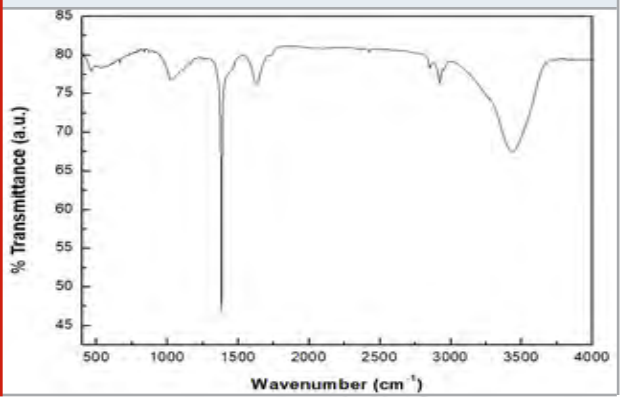
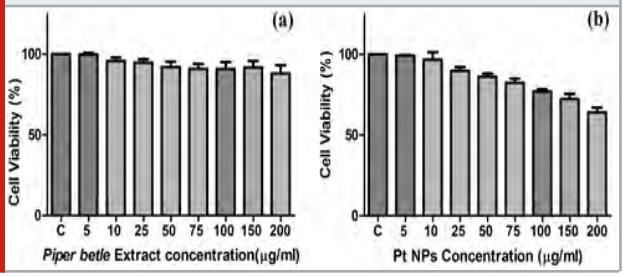


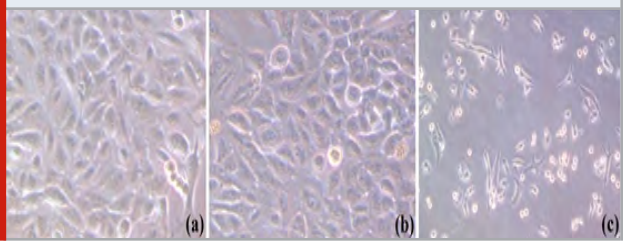
Figure 4: Cytotoxicity of (a) *Piper betle* leaves extract and (b) PtNPs on lung cancer cell line (A549).



The PtNPs surrounded by ethnomedicinal plant residues show intense and broad peak in the range of 3400 cm^{-1} with two peaks at 3693 cm^{-1} and 3448 cm^{-1} which owes to alcohol –OH stretching vibration and intramolecular H-bond. Polyphenol is the main metabolite present in the medicinal *Piper betle* leaves which is bestowed with phenolic hydroxyl groups (Tsao, 2010). The peak around 1780 cm^{-1} may be due to C=O bond of anhydride, aldehyde, ketone, ester, acid etc. of flavones, quinines. The band at about 1633 cm-1 is due to C=C stretching and 1513 cm^{-1} is owing to C=N stretching. The set of bands associated with some splitting; centered at 1600 cm^{-1} and 1500 cm^{-1} is due to aromatic ring vibrations (Coates, 2006). The fingerprint region 1500 cm^{-1} - 400 cm^{-1} has diverse vibrational peaks. The FTIR data communicates of the potential interaction of medicinal plant metabolites and the PtNPs that aid both in fabrication and functionalization.

Comparative analysis of cytotoxicity potential of plant extract and PtNPs: The MTT assay using lung cancer cell line (A549) of PtNPs was done along with that of plant extract with a range of dose concentrations for 24hrs duration in triplicates to check the cytotoxic efficacy of nanoparticles in comparison to that of the ethnomedicinal *Piper betle* plant extract. The cell viability data are expressed in Figure 4(a) and 4(b). The viability of cells for each concentration was compared to that of plant extract treated viability at the same dose. Both the data were presented as mean±SE of experiments done in triplicates. It was observed that PtNPs cutoff the growth of cancer cells significantly (***p<0.001) with inhibition of 36.01%of cells as compared to 12.06% (*p<0.05) of cells killed by plant extract at the maximal dose of 200µg/ml. It was seen that the reduction in cell viability by both the plant extract and the PtNPs is directly proportional to the dose increment.

Figure 5: Morphological alterations of lung cancer cell line (A549) (a) untreated cells, (b) treated with *Piper betle* leaves extract, and (c) treated with PtNPs synthesized form *Piper betle* leaves extract.



The cell viability after PtNPs treatment at the dose of 5µg/ml was 99.35% (difference not significant p>0.05), at 10µg/ml was 96.75% (difference not significant p>0.05), at 25µg/ml was 89.77% (**p<0.01), at 50µg/ml was 86.11%(**p<0.01), at 75µg/ml was 82.34% (**p<0.01), at 100µg/ml was 77.05% (***p<0.001), at 150µg/ml was 72.31% (***p<0.001) and at 200µg/ml was 63.99% (***p<0.001) as compared to 99.81% (difference not significant p>0.05) at the treatment dose of 5µg/ml, 95.82%(difference not significant p>0.05) at the dose of 10µg/ml, 94.68% (difference not significant p>0.05) at the dose of 25µg/ml, 92.06% (*p<0.05) at the dose of 50µg/ml, 90.89%(*p<0.05) at the dose of 75µg/ml, 90.74% (*p<0.05) at the dose of 100µg/ml, 91.66% (*p<0.05) at the dose of 150µg/ml, 87.94% (*p<0.05) at the dose of 200µg/ml of the *Piper betle* plant leaves extract. The results showed that different concentrations of PtNPs and plant extract have varied cytotoxic effects on A549 cell line.

The cytotoxicity increased with the increase in the dose concentration (Mishra et al., 2012) and it was affirmed that both PtNPs and plant extract reduces the cell viability of cancer cells but at all the doses PtNPs

proved to be more efficient in their cytotoxic effect in comparison to the extract. The images captured by the phase contrast microscope at the highest experimental dose of 200µg/ml of plant extract and PtNPs (Figure 5) showed a noticeable effect on the cultured cells with alterations in the morphology of the cells as compared to control (untreated) cells owing to cytotoxicity of both plant extract and PtNPs. In comparison to the untreated cells the treated cells contracted, loss in adherence was observed leading to floating of cells in the media. Both plant extract and the PtNPs treated cells exhibited morphological alterations. The effect as observed was more significant for the PtNPs.

CONCLUSION

This study counts on the fact that exploration of medicinal plants in nanofabrication avenues has many persisting scopes. The therapeutic potentiality of nanoparticles has been a matter of contention since long. The nanometer dimension owned by the nano-entities and the biological components forecasts the synergism among them and is the basis for probable applications of these entities as nanomedicine. Cellular level experiments with A549 epithelial lung cancer cell line elucidated the therapeutic efficacy of biogenic PtNPs fabricated from the ethnomedicinal plant *Piper betle*. In the reference frame of plant extract PtNPs were more efficient. This inference opens up avenues to revolutionize the ethnomedicine in conjunction with nanotechnology as it is presumed that plant extract moieties provides some coating material to the bare nanoparticles which besides reducing toxicity add on therapeutic advantage to the synthesized PtNPs thereby reducing the side effects. The in vitro cytotoxicity effect of biologically fabricated and functionalized PtNPs against A549 as witnessed proves to be a leading-edge venture, foretelling the potentiality of these nanoparticles for cancer therapeutics. This work is a small step towards foreshadowing possible alternative therapeutics for cancer and revolutionizing our ancient ethnomedicine. It attempts to remould the ethnomedicine by conjunction with nanotechnology.

Authors Contributions: All authors have contributed equally in bringing out this research work.

Conflict of Interest: None.

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Indole 3-Acetic Acid Production by *Aspergillus* Species Isolated from Chilli *Rhizospheres*

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ABSTRACT

In the present study soil samples were collected from different chilli rhizospheres in the vicinity of Guntur, Andhra Pradesh, India and the filed trails were conducted in the year of 2018-2019. A total of 57 microbial strains were isolated from chilli rhizosphere. All the strains were isolated from potato dextrose agar media by using 10-4 serial dilutions. Fine and clear colonies were picked and transformed into a culture tubes for further studies. For the preliminary screening there are 10 isolates were considered as fungal strains based on spore morphology. The spores are round and irregular in shape with green to light brown in colour. Preliminary identification of these fungal isolates was based on morphological and cultural characters on potato dextrose agar medium. All ten strains showed the maximum indole acetic acid production on Czepakdox agar medium. Various optimization studies like incubation period, pH, temperature and carbon and nitrogen sources were studied by affecting the indole acetic acid productions i.e., 10 days incubation period, pH 7.0 and 30°C. Carbon and nitrogen sources are also affected the indole acetic acid production in this optimization. A carbon and nitrogen source in the optimal medium plays a major role for the production indole acetic acid. Among them, the strain *Aspergillus* PB-7 in presence of Glucose and peptone showed the maximum IAA production of 110µg/ml and 75µg/ml. Successful inoculation of agricultural crops with biocontrol plant growth promoters includes the delivery of sufficient inoculum to the target, economical production of large quantities of microorganisms. Rhizospheric microbes especially plant growth promoting microorganism were promising to be developed as multifunctional biofertilizer.

KEY WORDS: PLANT GROWTH PROMOTERS (PGP), INDOLE ACETIC ACID (IAA), POTATO DEXTROSE AGAR (PDA)..

INTRODUCTION

Chilli can be grown in a wide range of Black, Brown, Red and Clay soils, but black soils which retain moisture for long periods are suitable for rain fed crop whereas well drained soils, deltaic soils and sandy loams are good under irrigated condition. Whereas Sandy and loamy soils poorly supported to chilli production when compare to black soils. Various climatic conditions like humid and hot weather supports to the chilli plants. Sometimes environmental conditions are also adversely affected the chilli growth and fruit production. India is the largest producer and consumer of chilli among other major producers in the world. India contributes about 47 per cent to the total world production, and assumes first position in terms of international trade, exporting 38 per cent of its total production.

Chilli production in India is moving northwards on increasing demand from diversified sectors and changing consumption patterns. Dry chilli production rose by nearly 52 per by nearly 52 per cent from 9.7 lakh tones in 1997-98 to about 18 lakh tonnes. More than 80% of the

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bacteria isolated from the rhizosphere can produce (IAA) Indole Acetic Acid (Khalid et al., 2004) in the presence of precursor, tryptophan either through root exudates or from the proteins released by the dead bacteria cells (Patten and Glick, 1996). Indole-3-acetic acid (IAA), a plant growth hormone compound, is a natural auxin produced by plants, bacteria, fungi and a diverse group of organisms. It is a metabolite derived from tryptophan by many tryptophan dependant and tryptophan independent pathways in plants as well as bacteria and fungi. These growth improvers act as biocontrol agents. (Pattern and Glick, 2002; Wesam et al., 2017).

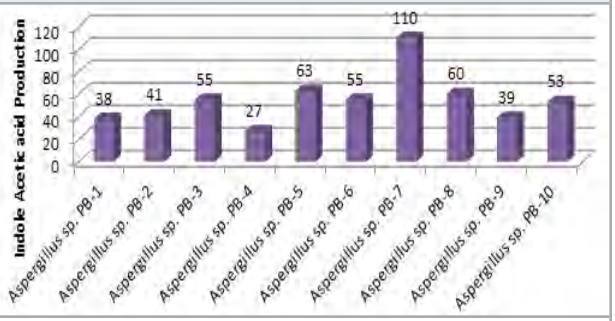
Various soil microorganisms including bacteria, fungi (Finnie and Van Staden, 1985) and algae (Stein et al., 1990; Rifat Hayat et al., 2010) are capable of producing physiologically active growth hormones like auxins and gibberellins which may exert prominent effects on plant growth and development. Many PGPR (Plant growth promoting) microorganisms associated in chilli rhizosphere. Chilli is one of the important agriculture produce Andhra Pradesh also. Chillies from Andhra Pradesh are well known for their pungency and good red colour. Several districts like Guntur, Krishna, Prakasam, Nellore, Chittor and Anantapuram are the main chilli growing districts in Andhra Pradesh. The present study was mainly focussed on indole acetic acid production and optimization studies by fungal strains isolated from chilli rhizosphere. These strains were used as bio inoculants in application to the farmer's fields and it is useful for sustainable agriculture (Wesam et al., 2017).

MATERIAL AND METHODS

Isolation of fungi done using the rhizosphere soil samples from the chilli fields of 10 different areas of Guntur, district of Andhra Pradesh, India were collected for the study. Fungal strains were isolated on Potato Dextrose Agar (PDA) medium by soil dilution plate technique (Rapilly, 1968) using 10^{-3} to 10^{-5} dilutions. The plates were incubated at 28 ± 2°C for 5 days. Fungal colonies appeared in the plates were noted and sub cultured. After purified by single spore isolation method and they were maintained on potato dextrose agar (PDA) slants. Identification of Fungal solates was based on culture characters as well as microscopic parameters (conidiophores branching, phialides shape and position, spore size and shape) (Nagamani et al., 2006). The pure cultures were stored in the refrigerator at 4°C for further studies.

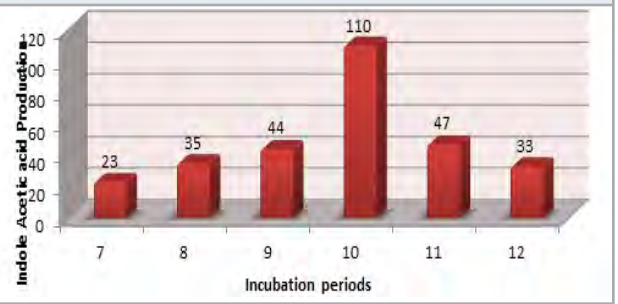
Screening of isolates for IAA production was determined based on the method described by Patten and Glick (2002) with slight modifications. One milli liter of supernatant was mixed with 2 ml of Salkowski reagent (1ml of 0.5M $FeCl_3$ in 50mL of 35% $HClO_4$) and incubated for 1hr. Development of pink colour indicated the production of IAA. The quantification of IAA was read at 540 nm in a UV- Vis spectrophotometer. A standard curve was plotted for quantification of IAA solution and uninoculated medium with a reagent as a control. The amount of IAA in the culture was expressed as µg/ml (Gordon and Weber, 1951).

Figure1: IAA production (µg/ml) by *Aspergillus* species



*The overall model is significant with $p<0.05$

Figure 2: Effect of incubation period on IAA production (µg/ml) by *Aspergillus* species PB 7



*The overall model is significant with $p<0.05$

The optimization studies for IAA production to determine the IAA production, different incubation periods (7, 8, 9, 10, 11 and 12 days), pH levels (4, 5, 6,7, 8 and 9) and temperature (4,20, 25, 30, 35 and 40°C) were studied. Various carbon (1%) and nitrogen (0.1%) sources were studied by using the above method.

For the statistical analysis, all measurements were carried out in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range tests. Differences at $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

A total of 57 strains were isolated from chilli fields in the vicinity of Guntur district, Andhra Pradesh, India. All the strains were tested for IAA production; among them 10 strains were positive results on Potato dextrose

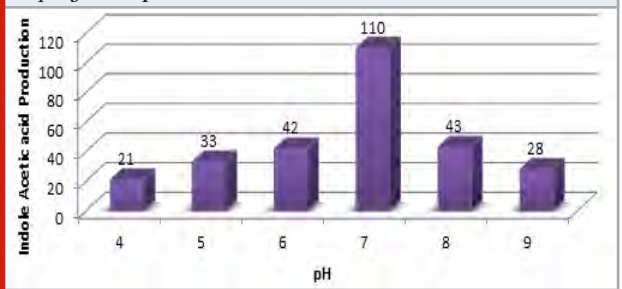
agar medium (Figure-1). The present study mainly reveals the IAA production of the selected 10 fungal strains. Maximum IAA production was observed on Aspergillus sp. PB 7 strain which showed 110 µg/ml of IAA, followed by *Aspergillus* sp. PB 5 strain (63 µg/ml). Minimum IAA production was recorded in *Aspergillus* sp. PB 4 with 27 µg/ml. IAA productions by ten fungal strains between the range of 27-110 µg/ml. *Aspergillus flavus* Promoted the Growth of Soybean and Sunflower Seedlings which was reported by Humayun et al., (2019).

Optimization studies for IAA production (µg/ml) by *Aspergillus* species PB 7: For the optimization studies we selected PB 7 *Aspergillus* strain which showed maximum IAA production and was tested with the effect of incubation period, pH and temperatures, carbon and nitrogen sources.

Effect of incubation period on IAA production by *Aspergillus* species PB 7: The IAA production was started between 7 to 12 days of incubation periods (Figure-2). The maximum IAA production was observed on 10th day of incubation (110 µg/ml). Similarly Unyayar et al., (2000) and Hansan (2002) reported that the maxumum amount of IAA was synthesized during the stationnary phase of growth. May be these reason was that during stationnary phase the bacterium might be able to get maximum tryptophan from dead bacterial mass, which could result in more IAA production. Reduction of IAA production at the later might be due to release of IAA degrading enzymes by the bacteria (Hunter, 1989).

Effect of pH on IAA production (µg/ml) by *Aspergillus* species PB-7: The pH of the medium showed a significant influence on the production of IAA by fungal strains. The maximum IAA production (110 µg/ml) was observed at pH 7 (Figure-3). For many authors, the optimum pH for IAA production is between 6 to 9. However, the below and above pH 8 the production of IAA was less, because Streptomyces sp population level is more in alkaline soil than the acidic soil (Shirokikh et al., 2007; Mohite, 2013; Bharucha et al., 2013; Dasri et al., 2014).

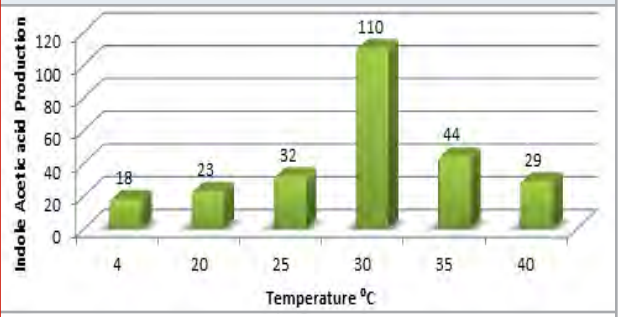
Figure 3: Effect of pH on IAA production (µg/ml) by *Aspergillus* species PB-7



*The overall model is significant with p<0.05

Effect of different temperature on IAA production (µg/ml) by *Aspergillus* species PB-7: The effect of different temperature on IAA production by fungal strains showed that, the maximum IAA production was obtained at 30°C (65 µg/ml) followed by 35°C (44 µg/ml) and 40°C (29 µg/ml) respectively (Figure-4).

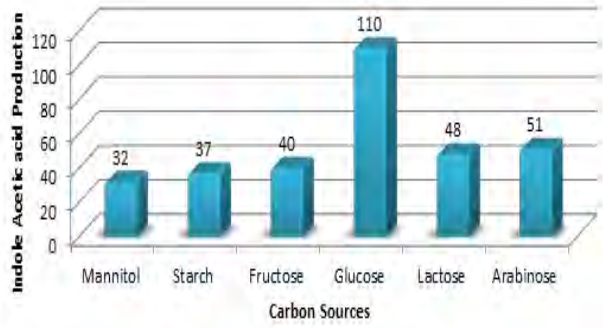
Figure 4: Effect of temperature on IAA production (µg/ml) by *Aspergillus* species PB-7



*The overall model is significant with p<0.05

Effect of different carbon source on IAA production (µg/ml) by *Aspergillus* species PB-7: Different carbon sources (mannitol, Starch, glucose, sucrose, fructose, lactose and Arabinose) were studied for their effect on IAA production by *Aspergillus* species PB-7 (Figure-5). Glucose in the medium gave maximum IAA production (110 µg/ml) followed by arabinose (51µg/ml) and lactose (48 µg/ml).

Figure 5: Effect of carbon sources on IAA production (µg/ml) by *Aspergillus* species PB-7

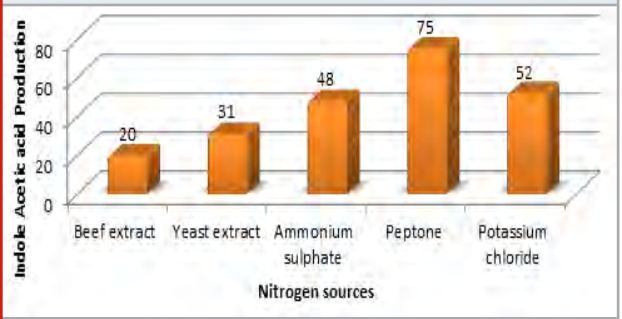


*The overall model is significant with p<0.05

Effect of different nitrogen sources on IAA production (µg/ml) by *Aspergillus* species PB-7: Various nitrogenous compounds (Ammonium sulphate, potassium chloride, Yeast extract, peptone and beef extract). These nitrogen source have a significant effect on IAA production (Figure-6). Among all the nitrogen sources used, peptone was found to be the best nitrogen source for IAA production. Organic and inorganic nitrogen sources can be utilized by *Pseudomonas* sp. prefers yeast extract (Balaji, 2012), *Pantoea agglomerans* PVM prefer beef

(Apine and Jadhav, 2011), and Ammonium sulphate was found to be for IAA production of *Acetobacter diazotrophicus* L1, the most suitable nitrogen source (Nita et al., 2011).

Figure 6: Effect of nitrogen sources on IAA production (µg/ml) by *Aspergillus* species PB-7



*The overall model is significant with $p<0.05$

CONCLUSION

From the results, it is clear that *Aspergillus* species PB 7 isolated form chilli rhizospheres preferred glucose and peptone as carbon and nitrogen sources respectively. The optimum conditions for this culture are incubation 12 days, pH 7.0 and 30°C temperature. There were several plant growth promoting rhizobacterial (PGPR) inoculants that seems to promote plant growth through different mechanism such as plant growth hormone production, nutrient acquisition and plant disease suppression. Thus the optimized microbial inoculants may be useful for the production of multifunctional biofertilizers.

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Suitability of *Melia composita* for Particleboard with the Replacement of Black Liquor and Phenol Formaldehyde Resin

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ABSTRACT

Wood in the form of timber and fuel wood it the most important product derived from the forest. Despite bestowed with over 4000 woody species a country like India is deficient in timber and there is a gap between supply and demand is burgeoning. *Melia composita* is a fast-growing timber species, the raw material for the study were collected and deliberately cut into pieces of 0.2 to 0.4 mm thickness, small length sundried to the moisture content of 8 to 12 percent. The sample was sieved through 60 microns for uniform size. Black liquor was collected from the Central Pulp and Paper Research Institute, Saharanpur, Uttar Pradesh. Phenol formaldehyde was prepared by mixing Phenol with formaldehyde solution. The formaldehyde solution with replacement of 10 percent Black liquor shows good results regarding the Indian standard IS: 2380-1977 i.e. average moisture content (5.35 %), volume (123.75 cm^3), density average (0.92 g/cm^3), water absorption % after 2 hrs (51.92), water absorption % after 24 hrs (64.10), swelling length (1.0), width (0.25), thickness (15.15), swelling % (22.82), MOR (16.15), MOE (1821.48), tensile strength (0.61), screw and nail withdrawal strength in the face (254.5), edge (247.5). . The findings show that the modification improves the particleboard quality especially the dimensional stability by using the modified resin for its manufacture.

KEY WORDS: BLACK LIQUOR, *MELIA COMPOSITA*, PARTICLEBOARD, PHENOL FORMALDEHYDE.

INTRODUCTION

Wood in the form of timber and fuel wood is the most important product derived from the forests. At present total forest cover and tree cover of the country are 21.67 % of its geographical area (Forest survey report, 2019). Despite being bestowed with over 4000 woody species the country is a timber deficient country and the gap between supply and demand is burgeoning. Shortage of wood raw material is being experienced in the country due to various reasons. This gap between demand and supply of various raw materials is likely to further widen due to the rise in population and standard of living. Because of this, some substitute raw materials for solid wood have been developed. There is a need to develop an alternate raw material base for all the wood-based industries including reconstituted panels. The trend is shifting to replace wood with alternative renewable lingo cellulosic materials that can efficiently meet the heavy demand.

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The assessment of durability through accelerated aging tests shows that panels protected with waterproofing material can be used in environments that have contact with moisture (Juliano et al. 2012). The demand for composite wood products particularly particleboard has been significantly increasing owing to their use in construction, cabinets, tabletops, vanities, sliding doors, speakers, table tennis, stair treads, kitchen work-tops, laboratories, and other industrial products (Alam et al. 2015). Particleboard offers a means to utilize as much as the forest and industrial wood waste as possible because it is so tolerant of wood quality and a wide variety of species, both soft-wood and hard- wood can be used. There is no doubt that the particleboard is going to stay for a long time due to plenty of raw materials, manufacturing properties and product properties (Biswal and Rout, 2015).

The manufacture of particleboard was introduced in India in 1957. The raw material used for such products can be from non-conventional sources like plantation under farm forestry, social forestry, wood residues and lignocellulosic residues of agricultural origin. Due to the shortage and increasing price of wood in the country, large plantation of commercially important fast-growing species of wood is being raised. In the future many more commonly found materials could very find its use in the manufacture of particleboard, thereby cutting down the costs drastically.

They also reduce the external pressure on the natural forest to save humankind. Particleboard has some advantages compared to solid wood; it is a homogenous material because the composite a well-mixed blend of reconstituted materials that give flexibility in size. However, it's presented some disadvantages also i.e. swelling due to water absorption. Particleboard used has some disadvantages, i.e. swelling due to the water absorption. Recently particleboard has begun to be used as a load bearing wall component in Japan. In addition to furniture materials. Researchers in the Middle East, South America and south East Asia have reported its usage as a constituent particleboard. The inefficiency of the solutions alternative less polluting, less toxic to replace conventional binder (Irmayanti et al. 2015).

Table 1. Physical Properties of the prepared resin

Phenol: BL	pH	Viscosity	Solid content	Ash content
100:0	12	5.10	12.24	18.78
90:10	12	5.5	11.79	16.56
80:20	12	5.40	11.86	17.70
70:30	11	4.80	12.15	17.30
60:40	10	6.96	12.50	18.50

Thus, the replacement of Black liquor and phenol formaldehyde gives the base to some great problem to achieve a good mechanical panel that is resistant to microbial agents. In this study, *Melia composita* was utilized to fabricate particleboard, and its performance was determined again after exposure to subterranean termite and wood decay fungi. Previous studies show the only contribution of density towards its termite resistance, (Suhasman et al. 2012). So, keeping in view the importance of the study the present work concerns the particleboard from *Melia composita* with the replacement of black liquor with Phenol Formaldehyde resin, (Utsumi et al. 2019).

Table 2. Minimum values of the parameters (IS: 3087:2005)

Sl. No.	Properties	Flat Pressed Single Layer
1	Density Variation, percent	±10
2	Water Absorption, percent	
a)	2h soaking	25
b)	24h soaking	50
3	Linear Expansion (Swelling in water), 2h soaking, percent	
a)	Length	0.5
b)	Width	0.5
4	Thickness swelling, percent,2h soaking	10
5	Swelling in thickness due to	
	surface absorption, percent	9
6	Modulus of rupture, N/mm2	
a)	Average	11
b)	Minimum	10
7	Modulus of elasticity, N/mm2	
a)	Average	2000
b)	Minimum	1800
8	Tensile Strength perpendicular to Surface, N/mm2	
a)	Up to 20mm thickness	0.8
b)	Above 20mm thickness	0.8
9	Screw withdrawal Strength, N	
a)	Face	
b)	Edge (foe thickness > 12mm)	1250
		850

MATERIAL AND METHODS

Melia composita is taken as raw material largely distributed in moist teak forests, secondary moist deciduous forest and dry mixed moist deciduous forests of India, like Sikkim Himalayas, North Bengal, Upper Assam, Khasi Hills, hills of Odisha. It is a large tree, attaining a height of 20 m. with a spreading crown and a cylindrical straight bole of 9 m. length and 1.2-1.5 m. girth (Khare, 2007). The required material of wood was obtained from the Forest Research Institute campus, Dehradun, India and was deliberately made into small pieces with the help of sickle. Then the small pieces were

passed through the Condux mill to get particles of 0.2 to 0.4 mm thickness and smaller length. Then the particles were sun dried to a moisture content of 8 to 12%.

Then the particles were sieved through a 60-micron mesh to obtain uniform particle size (Table.1). Black liquor (BL) is a complex solution containing lignin as well as its degradation products, polysaccharides, extractives and several inorganic compounds (Stenius, 2000). Black Liquor already had been effectively used as an adhesive for wood composites in various researches (Singh and Joshi, 1990). The Black Liquor that was used for the research purpose was collected from Central Pulp and Paper Research Institute, Saharanpur which was crafted from North-East bamboo (Kouisni et al. 2011).

Table 3. Details of Particleboard

Board No (Sample No)	Resin Content	Pressure used	Resin content used
1	10%	21Kg/cm^2	90% Phenol+ 10% BL
2	10%	21 Kg/cm^2	80% Phenol+ 20% BL
3	10%	21 Kg/cm^2	70% Phenol + 30% BL
4	10%	21 Kg/cm^2	60% Phenol + 40% BL.
5	10%	21 Kg/cm^2	100% Phenol+ 0% BL

The Phenol-formaldehyde resin (PL) was prepared by mixing Phenol with formalin solution. For that, 1000 ml of phenol was taken in a round bottom flask. Then 1200 ml of formalin and 1200 ml of water were added to the flask. The pH of the whole solution was maintained at 9-9.5 by adding 50 g. of NaOH dissolved in 100 ml. of water. The round flask with a solution was mounted on a boiling water bath and observed untill the actual reaction started inside the flask. The actual reaction started when the first time the bubbling was seen in the solution. The solution was refluxed for 30 min. The resin was kept for cooling. The cooled resin was analyzed for suitability in making particleboard. Preparation of Modified Resin with Black Liquor done using exactly 0, 10, 20, 30 and 40% (w/w) black liquor was added to PF resin (PF-BL). The mixture (PF resin and black liquor) was compounded using a mechanical stirrer at 3000 rpm, for 15 min. The BL-PF was employed to analyze and characterize the properties of this adhesive like pH, solid content, Ash content and viscosity (Kouisni et al. 2011).

The PF-BL particleboards were prepared by blending the BL-PF resin with the dried particles in a rotating drum type mixture fitted with a pneumatic gun spray. A total of 10% of resin to the total dry weight of particle for each board was used for the spray. All boards were compressed in the hot air pressure at 21.0 Kg/cm^2 pressure at a temperature of 150 ^{o}C for 15 minutes. The dimensions of each board were fixed at a dimension of 24 x 24 inches for laboratory testing and then were conditioned for 2-3 days at room temperature before converting to testing samples.

Table 4. Moisture Content and Density of Particleboards

Sl. no	Sample No.	Initial Wt (Mi)(g)	Oven Dry Wt (Mo)(g)	M.C. %	M.C. % Avg.	Volume (cm^3)	Density (g/cm^3)	Density Avg. (g/cm^3)
1	1a	88	83	5.95	5.35	123.75	0.93	0.92
	1b	88	84	4.76		121.28	0.91	
2	2a	76	72	5.55	5.39	123.75	0.93	0.87
	2b	74	69	7.24		121.28	0.91	
3	3a	77	71	8.45	5.40	101.25	0.71	0.83
	3b	81	76	6.57		101.25	0.73	
4	4a	76	72	5.55	6.39	111.00	0.91	0.78
	4b	74	69	7.24		100.57	0.83	
5	5a	90	71	8.45	7.51	99.23	0.87	0.72
	5b	85	76	6.57		111.00	0.79	

Two samples from each board were prepared for testing the physical and mechanical properties of the particleboards according to Indian standard IS: 2380-1977.The obtained results from each testing were compared with the minimum requirement for various physical and mechanical properties as per IS 3087:2005.

Indian Standards of Particleboards: The minimum requirement of particleboards for various physical and mechanical properties is given below in Table 2. Minimum requirement according to IS 3087:2005.

RESULTS AND DISCUSSION

Total 3. of boards were prepared using *Melia composita*

and Phenol resin with 10% replacement at each level under 21 Kg/cm^2 pressure for 15 minutes at 150 ^{0}C. The details of the boards prepared were given below.

Physical Properties: The values of various physical properties i.e. moisture content, density, water absorption, swelling percent and swelling due surface absorption of particleboards from *Melia composita* with Phenol resin with the replacement of 10% Black liquor with 10% resin content are depicted below in the table No.7, 8, 9 and 10 respectively. The values were calculated as per IS Specification (Fig.1). The findings are in accordance with the exiting standard and show the sign that *Melia composita* is suitable. The findings are in concurrence with the results found by (Villeneure 2006).

Figure 1: Moisture content (%) of the boards

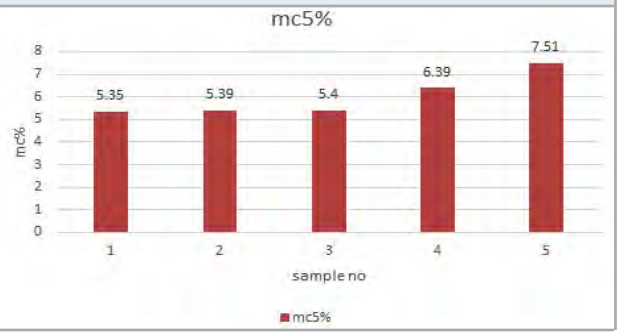
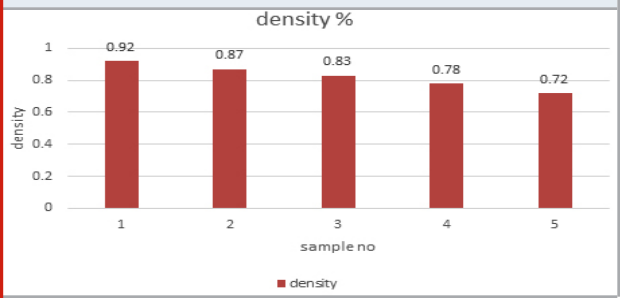


Figure 2: Average Density (%) of the boards



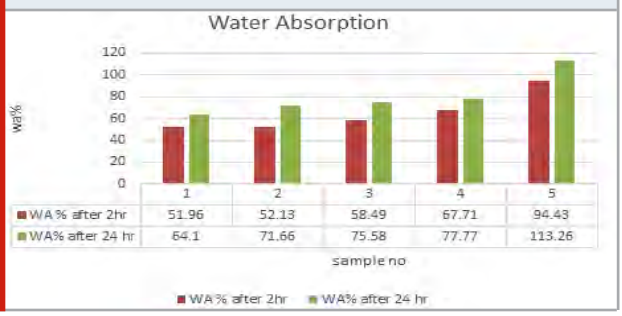
In the data obtained for moisture content of the particleboards, the average value of moisture content varies from 5.24% to 7.5%. Board no. 5 showed the highest moisture content % whereas Board no. 1 showed lowest one (Fig.2). Particleboard used the modified adhesive for the construction results in good performance for the furniture purpose. The findings where in conformity with the findings of (Biswal and Rout 2015).

In the data obtained for density of the particleboards, the mean value of density varied from0.70 g/cm^3 to 0.95 g/cm3.Highest density was showed by the board no. 1 whereas board no. 5 showed the lowest one. Increase in the density represents high mechanical properties.

Table 5. Water Absorption of particleboards from *Melia composita* and Phenol resin with 10% replacement of Black liquor

Board No	Sl.No	Initial Wt(g)	After 2hr Wt(g)	After 24hr Wt(g)	WA% after 2hr	WA% after 2hr Avg.	WA% after 24hr	WA% after 24hr Avg.
1	1a	133	219	246	64.66	51.92	84.96	64.10
	1b	132	213	238	61.36		88.33	
2	2a	121	194	216	60.33	52.13	78.51	71.66
	2b	121	202	222	56.94		83.47	
3	3a	142	213	237	50.00	58.49	66.90	75.58
	3b	137	215	236	56.93		72.26	
4	4a	114	182	204	59.64	67.71	78.94	77.77
	4b	140	210	230	50.00		64.28	
5	5a	150	238	258	58.66	94.43	72.00	113.26
	5b	158	233	252	84.74		59.49	

Figure 3. Water Absorption of the Boards



The results were in accordance with the findings of Wang (2002) for convention pressing time 5-15 Min at a pressure of 10 to 30 Bar. In water absorption, all the board from shows higher value than the critical value for 2hr and 24 hr as per IS 3087:2005 (Fig.3).

In general swelling, the board No. 1 shows the lowest value for width which nearly to the controlled condition. Board no. 1 and 5 are also quite nearer to each other in case of swelling in its thickness. No board for thickness swelling is within the critical value for 2 hr soaking as set in IS 3087:2005. As for other boards made from different material ratio, the linear and thickness swelling

values were quite high. The results are slightly in conformity with the existing standard and show *Melia compostita* can be used in outdoor environment. There is a possibility of the mechanical properties being affected by the swelling of the particleboard (Fig.4) (Utsumi et al. 2019).

Table 6. Swelling percent of particleboards from *Melia composita* and Phenol resin with 10% replacement of Black liquor

Board No	Sl.No	In Length (mm)	Length Avg.	In Width (mm)	Width Avg.	In Thickness (mm)	Thickness Avg.
1	1a	1.00	1.00	0.30	0.25	10.0	15.15
	1b	1.00		0.20		8.0	
2	2a	1.00	1.00	0.50	0.35	13.9	14.95
	2b	1.00		0.20		16.0	
3	3a	1.00	1.00	0.80	0.90	14.0	14.6
	3b	1.00		1.00		15.2	
4	4a	0.50	0.75	0.50	0.75	13.2	13.5
	4b	1.00		1.00		13.8	
5	5a	1.00	1.00	1.00	0.80	12.2	12.9
	5b	1.00		0.60		13.6	

Figure 4: Linear expansion of the board samples.

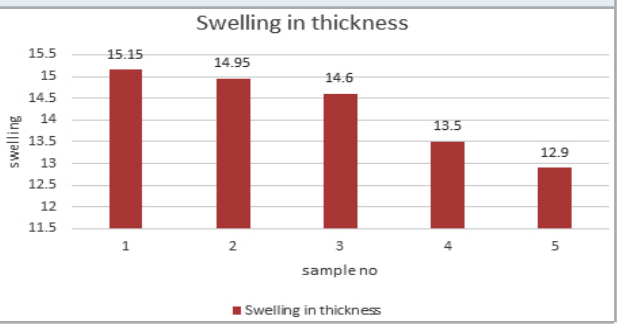


Figure 5: Surface swelling of the board

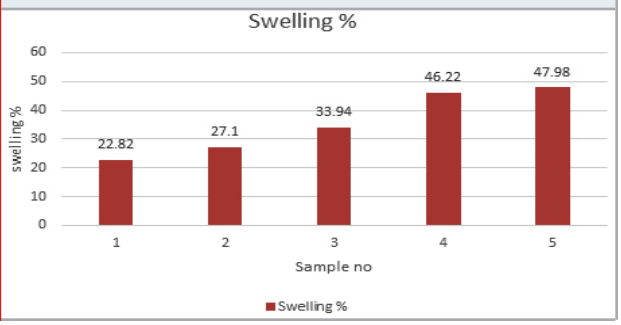


Table 7. Swelling due surface absorption of particle boards from water absorption of particleboards from M*elia composita* and Phenol resin with 10% replacement of Black liquor

Board No	Sl.No	Initial Thickness Avg. (mm)	Thickness after 2hr Avg. (mm)	Swelling after 2hr (mm)	% Swelling after 2hr	% Swelling Avg.
1	1a	10.68	13.02	2.34	21.91	22.82
	1b	10.95	13.55	2.60	23.74	
2	2a	10.01	12.57	2.56	25.57	27.10
	2b	10.30	13.25	2.95	28.64	
3	3a	10.55	13.80	3.25	30.80	33.94
	3b	10.92	14.97	4.05	37.08	
4	4a	10.18	14.67	4.49	44.10	46.22
	4b	10.36	15.37	5.01	48.35	
5	5a	9.65	13.65	4.00	41.45	47.98
	5b	10.27	15.87	5.60	54.52	

In surface swelling, all the boards are above the critical value as per IS 3087:2005. Board 1 shows nearly equal value with Board 5 while from Board 2 to 5 there is increasing order in swelling (Fig.5).

Table 8. Static Bending Strength of particleboards from *Melia composita* and Phenol resin with 10% replacement of Black liquor

Board No	Sl.No	Max. Load N	MOR (N/mm²)	MOR Avg. (N/mm²)	MOE (N/mm²) after 2hr	MOE Avg. (N/mm²)
1	1a	389.5	16.99	16.51	1945.60	1821.48
	1b	362.50	16.03		1697.37	
2	2a	315.00	13.93	13.47	1521.32	1461.97
	2b	294.50	13.02		1402.63	
3	3a	291.50	12.89	11.89	1242.97	1142.42
	3b	224.00	10.89		1041.88	
4	4a	198.00	10.59	9.94	1141.73	1073.93
	4b	191.00	9.29		1006.13	
5	5a	183.50	8.92	7.42	910.13	829.78
	5b	134.00	5.92		749.44	

Figure 6: Modulus of Elasticity of the board samples

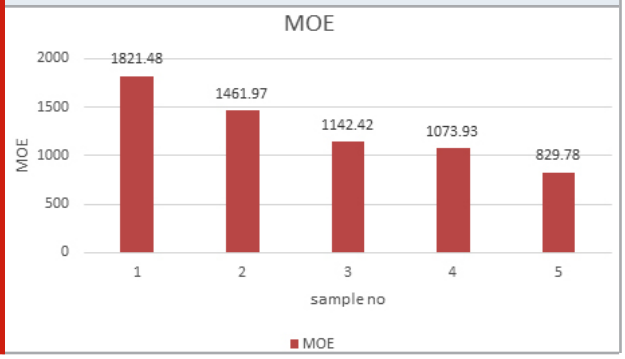


Figure 7: Modulus of Rupture of the board samples

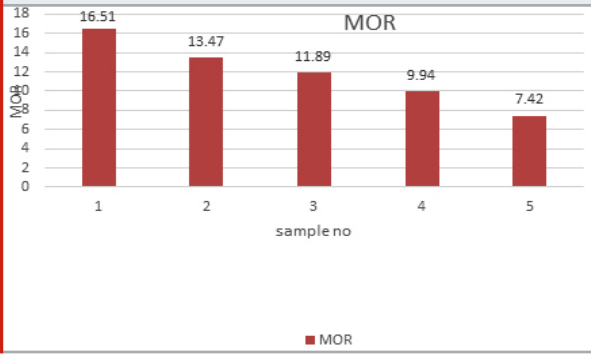


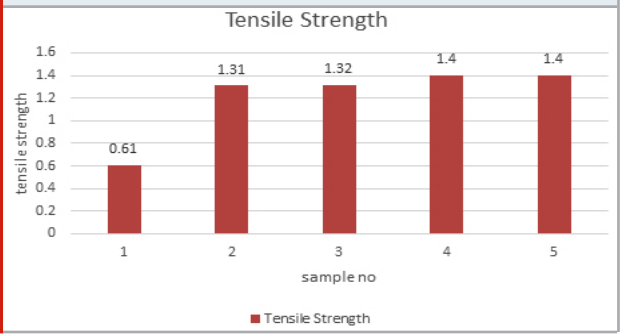
Table 9. Tensile Strength Perpendicular to Surface of particleboards from *Melia composita* and Phenol resin with 10% replacement of Black liquor

Board No	Sl.No	Length (mm)	Width (mm)	Area (mm²)	Load (kg)	Load (N)	Tensile Strength (N/mm²)	Average Tensile Strength (N/mm²)
1	1a	50.5	50.8	2565.40	175	1715.0	0.66	0.61
	1b	50.7	51.3	2600.91	150	1470.0	0.56	
2	2a	51.0	50.5	2575.50	378	3704.4	1.43	1.31
	2b	49.6	49.7	2465.12	302	2959.6	1.20	
3	3a	51.7	50.6	2616.02	319	3126.2	1.19	1.32
	3b	51.1	50	2555.00	380	3724.0	1.45	
4	4a	50.4	51.4	2590.56	384	3763.2	1.45	1.40
	4b	51.4	51.2	2631.68	364	3567.2	1.35	
5	5a	50.4	51.4	2590.56	384	3763.2	1.45	1.40
	5b	51.4	51.2	2631.68	364	3567.2	1.35	

Mechanical properties: Mechanical properties i.e. static bending strength, perpendicular to surface and screw and nail withdrawal strength of particleboards from Melia composita and Phenol resin with 10% replacement of Black liquor were depicted below in table no.6, 7, 8, 9 and 10 respectively (Utsumi et al. 2019).

MOR and MOE values were the highest for board no. 1 which were 16.51 N/mm2 and 1821.48 N/mm^2. Board no 5 failed to satisfy MOR value as per requirements of IS-3087-2005.

Figure 8: Tensile Strength of the board samples



Internal bond strength depends upon the particle and interaction with the adhesive. In internal bonding, the board 4shows value 1.4 N/mm^2 which is the maximum obtained among all the boards. Furthermore, the internal bond strength value decreases in the case of boards prepared with different material ratios. All Boards except board 2 from the after study all other boards didn't meet the specification of IS.3087:2005. The possibility of the results may be due to the shape of the chips and the combination of Black liquor and Phenol formaldehyde. The values were in confirmation with the results found by Kadja (2012) on Kenf particleboard with bone glue beads.

Sample 2 of the boards made from *Melia composita* showed the maximum values i.e. 2059.39 N/cm^2 for face and 1853.45 N/cm^2 for edge for screw withdrawal resistance respectively. The samples from boards 3 and 5 also show good results but a little less to meet the requirements of specification of IS 3087:2005. The lowest face screw withdrawal resistance was found for board no. 1 and for the same edge screw withdrawal resistance also. In the earlier study on particleboard of *Melia azedarach* modified adhesive was prepared using Urea formaldehyde resin adhesive.

Table 10. Screw and Nail Withdrawal Strength of particleboards from Melia *composita* and Phenol resin with 10% replacement of Black liquor

Board No.	Sl.No.	Load (Kg/cm^2) Face Screw 1	Screw 2	Avg (kg)	Edge Screw 1	Screw2	Avg
1	1	224	285	254.5	232	263	247.5
4	1	247	258	252.5	201	177	189
5	1	271	224	247.5	194	180	187
2	1	230	259	244.5	160	184	172
3	1	229	227	228	176	153	164.5

Figure 9: Screw Withdrawal Strength of the board samples

These findings are in alignment with the findings of Yang et al. (2007) in wood waste chips impregnated with phenol formaldehyde resin.

CONCLUSION

The particleboards prepared from *Melia composita* using Phenol resin with 10% replacement of Black liquor resin with a resin concentration of 10%. The used replacement of Black liquor resin with Phenol is 10%, 20%, 30%, 40%, and control of 100% Phenol resin. The boards are prepared at a pressure of 21.5 Kg/cm^2 for 15 minutes of hot pressing. Based on a comparative analysis of the results, it is observed that there was an increase in the moisture content of the particleboards with increasing the percentage of Black liquor with Phenol resin. From the study, it can be concluded that 10% Black liquor can be used with the Phenol resin as a replacement in the making of particle board from *Melia composita* species.

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Proposed Framework to Build Information Technology Infrastructure for Sharing of Knowledge

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ABSTRACT

The current research project proposes a framework that can be used for building IT infrastructure for sharing of knowledge, with particular emphasis in the gulf region. The research project is important as currently, there is scarcity of the research projects addressing similar issues especially for the KM based infrastructure in the gulf region. Previously, authors have demonstrated that an effective approach of sharing knowledge is with IT. There are only a limited number of research works addresses KM infrastructure for the gulf region. To achieve this, the project effectively answers two research questions: what are the existing studies regarding IT and knowledge sharing from the gulf perspective. In addition, what framework, based on IT and knowledge-sharing concepts, can be used to build IT infrastructure for sharing of knowledge? The IT infrastructure can find a way of easing the sharing of tacit knowledge in much the same way as it eases the sharing of explicit knowledge, by demonstrating what makes the knowledge valuable, subsequently capturing this tacit knowledge in an explicit way, which can be achieved via the mode of externalization (tacit knowledge to explicit knowledge). The research used the systematic literature review as the research methodology to study the research objectives initially set from carrying out the study on past studies. In coming up with the IT infrastructure it is important to consider unique needs of an organization, the ease of use of the IT infrastructure for SOK, the availability of the technology as well as costs related to the technology. This report proposed a framework to build IT infrastructure for sharing of knowledge in the gulf region after considering the various findings.

KEY WORDS: FRAMEWORK; IT INFRASTRUCTURE; SHARING OF KNOWLEDGE; GULF; MIDDLE EAST.

INTRODUCTION

Knowledge management (KM) became a mainstream business management tool only during late 80s but gained prominence worldwide only after globalization. As the globalization allowed economic, social and technological changes to improve gradually, the knowledge management is seen as a tool to connect the branches worldwide with the knowledge gathered. Initially originated as a theory, the KM experts had many issues when they try to convert the theory into a working model. After, the widespread of internet and intranet, the implementation of the KM became easier (Antunes and Pinheiro, 2020; Ode and Ayavoo, 2020). Knowledge is an important strategic resource for individuals, groups and organizations. Knowledge can provide individuals, groups and organizations with some sustainable competitive advantages within today's highly competitive economy (Alaffad & Masrom, 2017).

One way to do this is through the information technology (IT) infrastructure within such organizations. Today, many organizations in the gulf have been undertaking

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some considerable initiatives for ensuring knowledge sharing become embedded in their organizations and in the day-to-day operations (Albassam, 2019; Dirani & Hamie, 2017). For example in the United Arab Emirates (UAE), government and the private sector have continued collaborating with the various western-based institutions in attempts at improving SOK in the region (Alshamsi & Ajmal, 2018).

In the gulf, adoption of processes for knowledge sharing has been partly motivated by establishment of the western based organizations in the gulf region (Youseff et al., 2017). In that manner, western-designed best practices and methodologies of sharing knowledge have been subsequently employed by gulf organizations. In gulf region, there have been efforts to do this and plans have been forwarded for ensuring the gulf citizens have active roles in this knowledge economy (Al-Ahbabi, 2017). The current research project proposes a framework that can be used for building IT infrastructure for sharing of knowledge, with particular emphasis in the gulf region. In many developed region across the globe, processes regarding sharing of knowledge (SOK hereafter) have been relatively well developed compared to the gulf region, owing to use of E-learning and technology (Akhavan & Mahdi Hosseini, 2015). Adopting such an approach in the gulf has often been gradual, with much emphasis that research institutions (Skaik & Othman, 2015) should undertake this.

For example in the United Arab Emirates (UAE), government and the private sector have continued collaborating with the various western-based institutions in attempts at improving SOK in the region (Alshamsi & Ajmal, 2018). However, using the western-designed best practices and methodologies of SOK has come with a price, as there is little emphasis on the peculiarities of the gulf region and way of life (Skaiak & Othman, 2015). Moreover, a recent research project that was funded by the European Union has indicated that within the gulf countries, the trends of IT are starkly different as a result of different development levels within and between the gulf countries (Göll & Zwiers, 2018). Despite that, the levels of IT adoption have continued to be pervasive in the gulf, and the increasing prevalence of IT in the region offers an opportunity to design infrastructure for sharing of knowledge (Göll & Zwiers, 2018).

To achieve this, the project effectively answers two research questions: What are the existing studies regarding IT and knowledge sharing from the gulf perspective? What framework, based on IT and knowledge sharing concepts, can be used to build IT infrastructure for sharing of knowledge? The outcomes of the current research project would not only be insightful to academics but also to the practitioners based in the gulf. Moreover, the study is significant as it fills the gap that currently exists in the present body of research work owing to lack of studies on similar topic. Overall, this contributes to the body of knowledge particular to the gulf region. A significant limitation of the project lies in the methodology that has been used.

The literature review methodology in use is convenient for the purposes of the research scope, and has overcome the impracticality of gathering primary data from the various gulf countries that have been considered in the framework. However the limitation of relying on the literature review methodology is that it has put the investigator at a disadvantage of not having the benefits of primary data collection, such as loss of control over the research process, and the loss of ability to get the most current data that would have been enhanced by primary research methods (Bryman, 2016).

MATERIAL AND METHODS

Definitions: For the purposes of the present research project, the operational definition is concise, detailed and clear definition of the measures employed in the research. For present research project, having an operational definition is fundamental towards collecting the intended data. The operational definition is especially significant as decisions have to be made regarding whether the data collected are valuable or useless, to do away with potential confusion. For example, the data collection process would not be helpful if definition of the timely and relevant data is not given (Slife et al., 2016). In the data collection, it was essential that the investigator had consistent approach towards sourcing and collecting data. This means defining how the data is collected, to remove room for inconsistent and erroneous data. Having the detailed operational definition blocks out any ambiguity in the data collection (Slife et al., 2016).

The following operational definition has been adopted:
Characteristic of interest: Framework to build IT infrastructure for sharing of knowledge, concentrating in the gulf region.

Measuring instrument: Investigator would collect data from scientific sources present online and in the physical library. Test method: Data from at least 27 scientific sources would be collected and analyzed. Only recent sources published after 2015 would be considered. Decision criteria: Systematically review the data from the sources. Only published material after 2015 would be considered and unpublished material (such as manuscripts) or material published before 2015 would be excluded.

Methodology: Conducting a systematic literature review (SLR) was deemed highly appropriate for present project. The SLR involved identifying, selecting and subsequently critically appraising research so as to answer the two

earlier formulated research questions (Dewey & Drahota, 2016). The SLR followed clearly defined plan in which some criteria were stated prior to conducting the reviews, such as having specific requirements on the information that was searched and reported within a particular timeframe. To be more specific, the SLR employed in the current study considered research only from scientific sources (published journal articles, conference proceedings and white papers) which was based on issues linked to SOK, and preferably concentrating the studies on the gulf region (Kuwait, Oman, Saudi Arabia, UAE, Qatar and Bahrain) and wider Middle East region.

The SLR was highly convenient, and an advantage is that it assisted in weeding out possible sources with little value to add to present research. For instance, the investigator quickly skimmed through the abstracts and methodology of potential sources and if they showed no relation to the gulf context, they were excluded (Thome et al., 2016). The process used to identify the sources involved first accessing the online academic databases, such as the university database, for material on SOK. Keywords relating to the research topic (Information_Technology+Sharing_of_Knowledge+Gulf+Middle_East) were input in the search field, as well as the synonyms, as shown in table 1 below:

Table 1. Keywords plus synonyms

Keyword	Synonyms
Information Technology	IT, I.T, InfoTech
Sharing of Knowledge	Knowledge Sharing, Knowledge Management, Information Sharing
Gulf	Kuwait, Oman, Saudi Arabia, United Arab Emirates/UAE, Qatar, Bahrain
Middle East	MidEast
Source: Author's own	

The search outcomes revealed numerous academic and industry sources, including journal articles, white papers, and industry reports (Thome et al., 2016). To improve search outcomes, the search was limited to studies published post 2015. Based on recommendations of Atkinson and Oppenheimer (2016), a data repository had to be first established for the collected data, which linked the data to the two research questions earlier aforementioned. The purpose was to ease later analysis of such data. From the repository, the investigators first condensed the data.

This involved the selection, the aggregation and the simplification of data that the repository contained. In such data condensation, the investigator decided on some segments of the studies' findings which required coding, in addition to deciding the studies' findings which were not important, such as studies that were not written in clear English. Moreover, parts of the studies that were not deemed to be important (such as author's biography details, foreword and reflections) were eliminated. Such condensation sharpened, organized and focused the literature to be usable by the investigator (Thome et al., 2016).

After condensing the findings of literature, the investigator read and re-read the condensed material and subsequently generated initial codes, which were used to assign meaning to the segments of literature collected. This enabled the investigator to rapidly identify the segments linking to the two research questions. From the collected literature, the repeated observations that were made were given the codes. An example is that if Information Technology (IT) had impacts on Sharing of Knowledge (SOK), code that was assigned was 'IT-yes-SOK'. Alternatively, if Information Technology (IT) had no impacts on Sharing of Knowledge (SOK), then the assigned code was 'IT-no-SOK'. Different codes were assigned for all the patterns that could be observed in the literature, echoic the suggestions by Thome et al. (2016) on the issue of 'codes and coding'. This was done until all the literature was coded, and patterns in the literature clearly identified. As this project did not involve use of primary research respondents, then consent was implied to have been given to the original research team, and research ethics were further observed by not attempting to identify respondents in the original research discussions.

Procedure followed to draw conclusion: The method of 'extended text' is commonly applied in displaying data. However, such data is usually bulky, and has a less rigorous structure when compared to the alternative of the partial ordered display (POD) method. This utilization of POD approach enabled the investigator to quickly link the identified segments using the codes, and subsequently link to the research questions. The literature that was earlier condensed was categorised, and findings of different studies compared. The investigator relied on Microsoft Access (MS Access) 2016 application for categirizing as well as comparing the literature findings, via matrices.

In spite of being assisted by technology, the onus of conducting the resaecrh still fell on the investigator, and care was taken not to cede the control of research process. Through a feature of 'relational database' offered by MS Access 2016 application, different segments of the literature collected and assigned codes were linked. It was thus possible to have clear perspectives on the different athors who shared same position. For example,

the relational data base showed the authors who had similar understanding on impacts of IT on SOK, and vice versa. Finally, to arrive at a conclusion, the investigator compared and contrasted the different literature segments obtained from different scientific sources, arriving at definite findings (Thome et al., 2016).

RESULTS AND DISCUSSION

In the gulf and the larger Middle East region, the explicit knowledge is often stored within knowledge management systems that can be accessed by the members of the organization, and IT plays a big role in this storage and retrieval (Al-Busaidi & Olfman, 2017; Hossain, 2015). The knowledge is usually arranged in terms of 'taxonomy', where the metadata is associated with particular knowedge; this helps in serching and retrieving such knowledge. Organizations can opt to use IT solutions having advanced search capabilities. On the other hand, interactive forums are used for accessing tacit knowledge, where people are connected to their colleagues in both informal and formal settings. However, this is not as common as the searching and retrieval of explicit knowledge. The design of the solutions for SOK were initially narrow in scope in the gulf, but have remained general enough so that many organiztions (both large and small) can comfortably apply them. Such general designs have allowed for newer solutions to be tested prior to implementing and/or impacting broader user groups (Skaik & Othman, 2015).

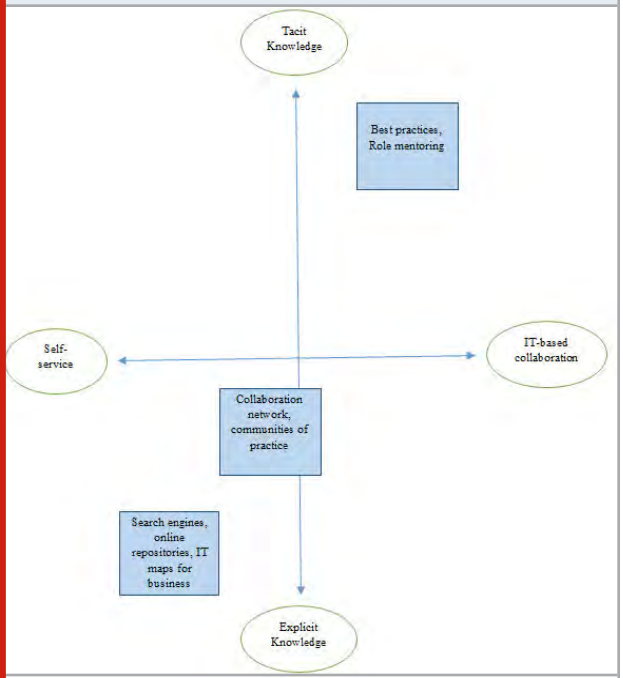
The literature has also revealed that there are scenarios in which widespread launching of SOK solutions could be desired, such as having centralized sites for whole organization to access experts, content and so forth, whih is still taking root in the gulf as the SOK concept is mostly a western concept. Authors, have also called for representatives of gulf businesses to be included in designing the SOK solutions. From the perspective of knowledge management, the early involvement by the future users can work well towards adopting more user-friendly solutions.

A close integration of the steps in the SOK with the actual steps undertaken in the organization's IT processes can geneate an overall unified structure that ensures members of the organization would routinely use the SOK solutiosn as an aspect of their everyday work lives. Whereas the goals could be deploying the SOK solutions and defining measures for effectiveness and use, am possible outcome is that the SOK solutions would be implemented with IT infrastructure support in placem (Al-Shamsi & Ajmal, 2018; Alaffad & Masrom, 2017).

A critical initial step towards the implementation of the SOK solutions is havimng a training plan and the relevant IT infratsructure support. As the members of the organization would be end users of the SOK solutions, training and testing an initial representative group could demonstrate the effectiveness of the IT infrastructure towards SOK (Chiabrishilli & Zaim, 2018; Obeidat et al., 2016). Training can subsequently be followed by close support which can include technical assistance and information sessions, especiallyn in the early implementation stages. In the implemnetation of the SOK solutions, the progress can be evaluated through active utilization of the IT infratsructure. Measures can include number of unique members/visitors as well as usimng the IT infrastructure to improve sharing of tacit knowledge.

As such, the leaders of the organizations can play big roles in reinforcing and modeling desired behaviours as well as utilizing SOK approaches (Hossain, 2015; Islam et al., 2015). Ongoing recognition of the desired SOK behaviours, supported by use of IT infrastructure, can promote this desired use of IT infrastructure for sharing of knowledge. However, there is need for clear accountability for the SOK solutions.

Figure 1: Proposed framework to build IT infrastructure for sharing of knowledge in the gulf region.



Source: Author's own

The IT infrastructure can find a way of easing the sharing of tacit knowledge in much the same way as it eases the sharing of explicit knowledge, by demonstrating what makes the knowledge valuable, subsequently capturing this tacit knowledge in an explicit way, which can be achieved via the mode of externalization (tacit knowledge to explicit knowledge). In coming up with the

IT infrastructure it is important to consider unique needs of an organization, the ease of use of the IT infrastructure for SOK, the availability of the technology as well as costs related to the technology (Skaik & Othman, 2015; Zaim, 2016). From the findings, a proposed framework to build IT infrastructure for sharing of knowledge in the gulf region is shown in figure 1 below:

CONCLUSION

For organizations and individuals to maintain competitive advantages, both organizations and individuals rely on systems, which are focused on selecting and imparting specific set of knowledge, abilities and skills. As knowledge is an important aspect, facilitating the generation, utilization and subsequent sharing of knowledge has become increasingly important today. The high importance in sharing explicit knowledge between the members of the organization, and making such knowledge available to each member, which also includes the new members of the organization who could refer back to the knowledge management systems for obtaining expert information.

The literature review findings indicated that there are some feasibility challenges about reliance on information technology for sharing the tacit knowledge. The research carried out on the Gulf nations with the help of the systematic literature review. After considering multiple aspects, the study here proposed a framework to build IT infrastructure for sharing of knowledge in the gulf region. It is also recommended that future research building can build on the findings of this study, but can also include primary data to make the findings more robust.

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Response of Rabi Maize to Different Methods of Irrigation and Fertigation

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ABSTRACT

A field experiment was conducted on maize for three cropping seasons during rabi 2008-09, 2009-10 and 2010-11 in strip plot design with 3 replications at Water Technology Centre farm, College of Agriculture, Rajendranagar, Hyderabad to determine the best irrigation scheduling and fertilizer level through drip fertigation to optimize maize grain yield and water productivity. The treatment consisted of four irrigation methods and schedules as main treatments (surface furrow irrigation at 1.0 IW/CPE, drip irrigation at 0.7, 1.0 and 1.2 Epan and three fertigation levels (150, 100 and 75% NPK Recommended Dose of Fertilizer (RDF)) as sub treatments. The results showed that increasing the irrigation water quantity and the fertilizer level (NPK) increased the maize grain yield. The highest and the lowest grain yield were obtained under the drip fertigation scheduled at 1.2 and of 0.7 Epan, respectively. The fertilizer application with 150% RDF (NPK) resulted in higher grain yield than the lower doses of NPK (75% and 100% RDF). Application of 150% RDF (NPK) and drip fertigation at 0.7 Epan gave the highest water productivity (0.78, 0.91 and 1.05; 0.76, 1.01 and 1.13 kg m-3 in 2008-09, 2009-10 and 2010-11, respectively) closely followed by drip fertigation at 1.0 Epan (0.73,0.78 and 0.97 kg m^{-3} in 2008-09, 2009-10 and 2010-11, respectively). Drip fertigation regime at 1.0 Epan saved on an average of 22% of the water as compared to the control surface ridge and furrow irrigation (1.0 IW/CPE). From three years experimentation, it can be advocated for maize crop under semi arid conditions to irrigate crop at 1.0 IW/CPE through drip under limited water available conditions.

KEY WORDS: MAIZE YIELD, RIDGE AND FURROW IRRIGATION, DRIP FERTIGATION, WATER PRODUCTIVITY.

INTRODUCTION

Maize is grown in *kharif* (June to October) under rain fed and also under supplemental irrigation and in *rabi* (November to March) under irrigated conditions. The maize crop is irrigated with furrow or check basin where the irrigation efficiency seldom exceeds 50%. Being most exhaustive crop, maize extracts more nutrients and also responds well to the irrigation as it's one of the crops with higher yield potential. Since water and nutrients are the critical inputs for agriculture, their better management and effective utilization is very much essential for successful crop production and is also a serious challenge to future food security and environmental sustainability (Hanumanth et al., 2016 Shruthi et al., 2018).

Fertigation along with drip irrigation has gained importance in present day crop production as water is a limiting factor and major source for crop production. In India, the irrigation efficiency does not exceed 40%. Due to water scarcity, the available water resources are

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to be effectively utilized through adoption of water saving technologies. Under such situations, increase in water productivity has greater role. It has been observed that the productivity of maize increases with increase in fertilization (Reddy et al., 1980) without considerable increase in water consumption. This results in higher productivity of water.

Supply of nutrients to the crop at the time of maximum requirement is important. Supplying nutrients to the crop during growth period continuously helps in improving the efficiency and productivity of water and nutrients. The supply of nutrients in small quantity continuously at the root zone is possible through drip system of irrigation. With drip fertigation the nutrient application efficiency can be increased up to 90% (Dioudis et al., 2003; Payaro et al., 2006 Hanumanth et al., 2016). Further, it is very important to consider how much fertilizer to be given and when to give the fertigation and also the crop stage and its nutrient demand, thereby one can achieve higher water and nutrient efficiency in addition to higher yield and economic returns.

The traditionally grown maize crop is fertilized with NPK in the form of urea, SSP/DAP and MOP. While the entire P and one third of N and half the dose of K are applied as basal, the remaining N is applied in 2 equal splits at knee high stage and flowering and rest of the K is applied at flowering. However, these fertilizers are not efficient to be made through drip irrigation. Better availability of nutrient with water soluble fertilizers result in higher growth attributes at fertigation levels of 50 and 75% RDF through drip over 100% RDF through soil. Similar results were found by Kumar and Pandian (2010) and Muthukrishnan et al., (2011).

The recent research findings have also revealed an increased crop yield and fertilizer use efficiency with fertigation supplied with water soluble NPK fertilizers (El-Hendawy et al., 2008, Hanumanth et al., 2016 Shruthi et al., 2018). Keeping in view of the above, a trial was conducted to evaluate the performance of *rabi* maize at different irrigation and fertigation levels with conventional and water soluble fertilizers to know yield performance and water productivity of *rabi* maize in semiarid regions.

MATERIAL AND METHODS

The experiment was conducted at Water Technology Centre Farm, Agriculture College, Rajendranagar, Hyderabad during *rabi* seasons of 2008-09, 2009-10 and 2010-11. The soil was sandy clay loam in texture with pH of 8.19, E.C of 0.57 dS m^{-1} along with a field capacity of 14.82 %. The fertility status of experimental site was low in nitrogen (242 kg ha^{-1}), medium in phosphorus (37 kg ha^{-1}) and high in potassium (526 kg ha^{-1}). The experiment was laid out in strip plot design with three replications. The four main treatments consisted of combination of irrigation methods and schedules viz. surface ridge and furrow irrigation at 1.0 IW/CPE, surface drip fertigation at 1.2, 1.0 and 0.75 Epan and three sub-treatments viz. fertilizer doses (150, 100 and 75% of recommended NPK). The maize hybrid DEKALAB super 900M was sown on 3rd, 5th and 3rd November in *rabi* 2008-09, 2009-10 and 2010-11, respectively on flat bed in paired row (40/80 cm) under surface drip irrigation and in ridges and furrows under conventional method of irrigation.

The drip system was laid out at 120 cm lateral spacing and 40 cm between dripper spacing with discharge rate of 2lph. In all the treatment combinations a spacing of 60 cm x 20 cm was adopted. The recommended fertilizer level of (120-60-50 kg NPK ha^{-1}) was applied through conventional straight fertilizers in conventional irrigation. In drip irrigation treatments, the fertilizers viz. water soluble fertilizers 19-19-19 (NPK), Mono Ammonium Phosphate (12-61-0 NPK) and KNO3 (13-0-46 NPK) were used as fertigation at 8 days interval from 12 to 80 DAS. Irrigations were scheduled as per the treatments by taking decennial average evaporation data obtained from Agro-Climatic Research Centre, Agricultural Research Institute, Rajendranagar, Hyderabad. Irrigation was scheduled at 3 days interval in drip and 6 to 8 days interval in ridge and furrow method of irrigation.

The recommended agronomic practices and plant protection measures were adopted as and when required. Irrigation water was scheduled to each treated plot at a pre-determined pan evaporation replenishment factor after adjusting the effective precipitation received (CropWat) and the amount of water discharged was measured through water meter attached to the irrigation system control unit. The water productivity (kg m^{-3} of water) was calculated by following equation. WP = Y/ WA (IR+ER) Where, Y = Grain yield (kg ha^{-1}); WA (total water used in m^3) = IR (irrigation) + ER (effective rainfall). The data on yield and water consumed in the experiment were recorded and the grain yield was analyzed statistically and where ever the treatment differences were found significant (F test), the critical difference was calculated at 5% probability.

RESULTS AND DISCUSSION

Grain Yield: In all the years of study, the irrigation regimes did not influenced the maize grain yield significantly except during *rabi* 2008-09. Whereas, the fertilizer levels significantly influenced the grain yield in all the years of study and interaction effect between irrigation regimes and fertilizer levels studied were found significant only during *rabi* 2008-09.During *rabi* 2008-09 drip fertigation at 1.2 Epan has resulted higher grain yield (5.58 t ha^{-1}) over rest of the irrigation regimes and it recorded 20 % higher grain yield over ridge and furrow method of irrigation (4.64 t ha^{-1}). Significantly lowest grain yield was noticed with drip fertigation scheduled at 0.7 Epan (3.68 t ha^{-1}). With respect to fertilizer doses, with increase in recommended fertilizer dose (RDF) from 75 to 100 and 150 % increased the grain yield significantly and the maximum grain yield (5.56 t ha^{-1}) was recorded at 150 % RDF. Higher grain yield (5.97 t ha^{-1}) was realized

with drip fertigation at 1.0 Epan and with 150 % RDF and differed significantly over all other irrigation regimes and fertilizer doses (Table 1 and 2 and Fig 1).

may be due to the leaching of nutrients away from the root zone as a result of the increasing amount of the irrigation water applied under the conventional ridge and furrow irrigation, which produced low yield, on the hand, the treatment at 0.7 Epan resulted in the lowest grain yield and exhibited a 20.0 % decrease in the grain yield compared to the conventional surface ridge and furrow irrigation. This means that the growth of plants might have stressed under the treatment at 0.7 Epan due to insufficient water supply. The higher grain yield obtained at 1.0 Epan with 150 % RDF of water soluble fertigation might be due to increased nutrient dynamics in the root zone and in plants under drip fertigation as documented by Mmolawa and Or (2000).

Table 1. Maize grain yield (t ha^{-1}) as influenced by irrigation regimes and fertigation levels

Treatments **Irrigation regimes**	Fertilizer dose **75% RDF**	**100%RDF**	**150 %RDF**	Mean
	Rabi 2008-09			
Ridges and furrow method at 1.0 IW/CPE	3.42	4.95	5.56	4.64
Fertigation at 1.2 Epan	4.36	5.82	6.55	5.58
Fertigation at 1.0 Epan	3.92	5.31	5.97	5.07
Fertigation at 0.7 Epan	3.20	3.69	4.16	3.68
Mean	3.73	4.94	5.56	
CD (5%) for irrigations	0.21			
CD (5%) for fertilizers	0.26			
CD at 5% Irrigation X fertigation	0.34			
CD (5%) for fertigation for same level of irrigation	0.59			
	Rabi 2009-10			
Ridges and furrow method	4.58	5.21	7.50	5.76
Fertigation at 1.2 Epan	5.00	6.04	6.46	5.83
Fertigation at 1.0 Epan	2.71	7.08	7.70	5.83
Fertigation at 0.7 Epan	4.38	5.21	6.67	5.42
Mean	4.18	5.89	7.08	
CD (5%) for irrigations	NS			
CD (5%) for fertilizers	0.80			
CD at 5% Irrigation X fertigation	N.S.			
CD (5%) for fertigation for same level of irrigation	NS			
	Rabi 2010-11			
Ridges and furrow method at 1.0 IW/CPE	3.75	3.90	4.68	4.11
Fertigation at 1.2 Epan	3.60	4.28	4.90	4.26
Fertigation at 1.0 Epan	2.55	4.08	5.17	3.93
Fertigation at 0.7 Epan	2.47	3.85	4.71	3.68
Mean	3.09	4.03	4.86	
CD (5%) for irrigations	NS			
CD (5%) for fertilizers	0.69			
CD at 5% Irrigation X fertigation	NS			
CD (5%) for fertigation for same level of irrigation	NS			

Table 2. Influence of irrigation methods and schedules on water productivity of maize

Treatments	Grain yield (t ha^{-1})	Water consumed (mm)			Total volume (m3 ha^{-1})	Water productivity (kg m-3)
		Irrigation	Rainfall	Total depth (mm)		
	Rabi 2008-09					
Ridges and Furrow	4.64	840	12.6	852.6	8526	0.54
method at 1.0 IW/CPE						
Fertigation at 1.2 E pan	5.58	807	12.6	820.2	8202	0.68
Fertigation at 1.0 E pan	5.07	673	12.6	685.6	6856	0.73
Fertigation at 0.7 E pan	3.68	471	12.6	483.7	4837	0.76
Mean water consumed					7105	
	Rabi 2009-10					
Ridges and Furrow method	5.76	900	45	945	9450	0.61
Fertigation at 1.2 E pan	5.83	840	45	885	8850	0.66
Fertigation at 1.0 E pan	5.83	700	45	745	7450	0.78
Fertigation at 0.7 E pan	5.42	490	45	535	5350	1.01
Mean water consumed					7775	
	Rabi 2010-11					
Ridges and Furrow method	4.11	510	41	551	5510	0.74
Fertigation at 1.2 E pan	4.26	420	41	461	4610	0.92
Fertigation at 1.0 E pan	3.93	365	41	402	4020	0.97
Fertigation at 0.7 E pan	3.68	285	41	326	3260	1.13
Mean water consumed					4350	

Table 3. Influence of fertigation levels on water productivity of maize

Fertigation levels, % Recommended fertilizer dose	Grain yield (kg ha^{-1})	Mean water consumed (m3 ha^{-1})	Water productivity (kg m^{-3})
	Rabi 2008-09		
75	3730	7105	0.53
100	4940	7105	0.70
150	5560	7105	0.78
	Rabi 2009-10		
75	4180	7775	0.54
100	5890	7775	0.76
150	7080	7775	0.91
	Rabi 2010-11		
75	3090	4350	0.71
100	4030	4350	0.92
150	4860	4350	1.05

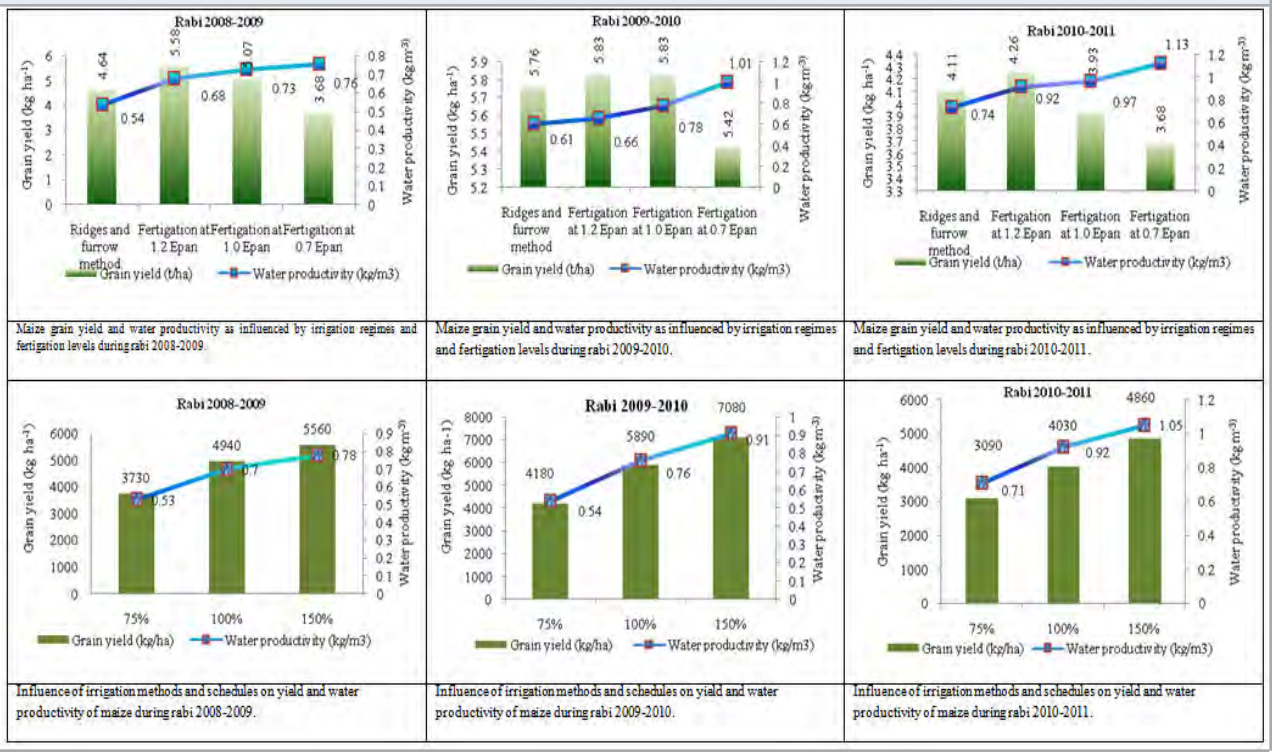
During *rabi* 2009-10, increase in fertigation levels from 75 % RDF to 100 and 150 % RDF has significantly influenced the maize grain yield. The highest grain yield (7.08 t ha^{-1}) recorded with 150 % RDF and differed significantly with rest of fertilizer doses. Similarly, during *rabi* 2010-11 also increase in fertigation levels from 75 % RDF to 100 and 150 % RDF has significantly influenced the maize grain yield. Fertigation with 150 % RDF recorded highest grain yield (4.86 t ha^{-1}) closely followed by 100 % RDF (4.03 t ha^{-1}). This may be due to the leaching of nutrients away from the root zone as a result of the increasing amount of the irrigation water applied under the conventional irrigation, which produced low yield, as shown in Table 4.

Meanwhile, the treatment at 0.6 Etc resulted in the lowest grain yield and exhibited a 46.4% decrement in the grain yield, compared to the control treatment. This means that the grown plants may be stressed under the treatment at 0.6 Etc due to insufficient water supply. The better growth of maize under drip might be attributed to better moisture availability, soil aeration and also

crop did not experience stress during the crop growth period at 1.0 Epan irrigation schedule. This ultimately reflected better physiological activity in plant and there by increased plant height and dry matter production in turn higher grain yield. Similar findings were reported by Tulu (1998).

Water Use: The total consumptive water use of maize in different irrigation regimes scheduled varied from 483 – 852, 535 -945 and 326 – 551 mm in 2008-09, 2009-10 and 2010-11 respectively. During *rabi* 2010-11, it was relatively warm and dry there by the actual crop evopotranspiration demand is more than the decennial average resulting lower yields as consequence of mild stress. In all the years of study the lowest total consumptive water use was associated with 0.7 Epan drip irrigation regime closely followed by drip irrigation at 1.0 Epan (Table 2). Drip fertigation regime at 1.0 Epan saved an average of 22 % of the water consumed compared to the control surface ridge and furrow irrigation (1.0 IW/CPE).

Figure 1: Pooled grain yield and water productivity as influenced by irrigation and fertilizer levels



Highest water productivity (0.76, 1.01 and 1.13 kg m-3 in 2008-09,2009-10 and 2010-11,respectively) was observed with drip fertigation at 0.7 E pan among the irrigation regimes (Table 2) which was closely followed by drip fertigation at 1.0 E pan. Among the fertilizer doses tested application of 150 % RDF of NPK (0.78, 0.91 and 1.05 kg m-3 in 2008-09, 2009-10 and 2010-11, respectively) resulted higher water productivity (Table 3 and Fig 1). Similar results were obtained by Zwart and Bastiaansen (2004), who reported water productivity values of 1.1-2.7 kg m-3 for maize crop. These finding indicate that it is essential to employ appropriate irrigation method for applying required amount of irrigation water and the fertilizers through the irrigation water under the drip irrigation system.

CONCLUSION

Irrigation scheduling under the drip fertigation is more efficient than the conventional surface ridge and furrow irrigation in maize crop in the sandy clay loam soil. For realizing optimum grain yields and the water productivity, it is recommended to irrigate the maize crop at 1.0 Epan for every 3 days and applying the recommended fertilizer dose of 150 % NPK through drip fertigation in semi arid conditions.

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Analysis on Inhibiting Pathogenic Activity of Fungi *Curvularia lunata* by Essential Oils

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ABSTRACT

Essential oils play a major role in the management of phytopathogenic fungal diseases and come forward as a replacement of chemical fungicides. Many of the medicinal and aromatic plants essential oils are used for its preservative, and pesticidal properties. The study of how essential oils affect the phytopathogenic fungal diseases is not widely studied by many researchers, specifically, the essential oils which we have taken. This compelled us to conduct an original research for *Ageratum conyzoides*, *Zanthoxylum armatum*, and *Mentha arvensis* and fungi *Curvularia lunata*. The essential oils of *Ageratum conyzoides*, *Zanthoxylum armatum*, and *Mentha arvensis*, were procured from fresh herbage sampled from the Lucknow region of India. However, *A. conyzoides* was also sampled from the Lakhimpur, Pantnagar, and Kanpur region of India. These plant-based essential oils were extracted by the hydrodistillation method and were stored for further studies. The antifungal activity was examined by the use of poison food techniques. Hundred percent of fungal (*Curvularia lunata*) growth inhibition showed by the use of 0.2 mg/ml and 8 mg/ml concentration of *A. conyzoides* and *Z. armatum* essential oils respectively. *C. lunata* is the major cause of leaf spot/leaf blight disease on economically important crops and causing heavy loss of economy. *M. arvensis* essential oil showed greater antifungal activity as compared to its major compound menthol and DMO via poison food technique. Overall, the study revealed the antifungal activity of essential oils against *C. lunata*. Moreover, the essential oil of *M. arvensis* shows the higher inhibition as compare to the menthol/DMO against the *C. lunata*.

KEY WORDS: AGERATUM CONYZOIDES; ZANTHOXYLUM ARMATUM; MENTHA ARVENSIS; CURVULARIA LUNATA; ESSENTIAL OIL.

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INTRODUCTION

Foliar region of plant provides the favourable habitat for epiphytic microorganisms, many of which are capable to influence the growth of pathogens. More than 12% reduction of world crop yield reported due to pre- and post-harvest diseases and FAO proposed that about 20 to 40 percent of global crop yields losses per year by the plant pest and diseases (Agrios, 1997; FAO, 2020). The fungal disease causes major yield reduction and economic losses in agriculture and forestry of the world.

Among them, the foliar disease is one of the major constraints. Medicinal and aromatic plants (MAPs) have been commercially cultivated as a source of important medicinal constituents and essential oil in various parts of the world but adversely affected by many fungal diseases in the humid season. Fungal pathogens infect MAPs under the favorable condition and produce a serious threat to their commercial cultivators. In the present scenario, the infection of *Curvularia* spp. (Singh et al. 2020). on the commercial fields of MAPs increases day by day which adversely affected the economic yield of the crops. *Curvularia* belongs to family Pleosporaceae, the largest family in the Pleosporales (Zhang et al., 2012; Hyde et al., 2013; Wijayawardene et al., 2014).

Leaf blight and leaf spot are the most common foliar diseases caused by the *Curvularia* spp., and posed to potential threats in many important crops. *Curvularia lunata* seems to infect a wide range of medicinal and aromatic crops such leaf spot of *Zatropha curcus* in Mexico, leaf blight of *Ocimum basilicum*, Leaf spot of *Mentha arvensis*, and leaf spot of *Acorus calamus* in India (Thakur et al., 1974; Cisnero-Lopez et al., 2012; Srivastava et al., 2015; Srivastava et al., 2019). The Indian trade of medicinal and aromatic products is approximately 120 million US$ per annum. India is the biggest producers of MAPs and potential supplier of essential oils to the world market (Tripathi et al., 2016).

The foliar diseases (leaf spot and leaf blight) cause the economic losses and reduced the shelf life and market values of the food commodities. Thus, it is essential to manage the foliar disease with biological features without any harm the quality of MAPs. *Ageratum conyzoides* L., is an annual herb belong to the family Asteraceae, also known as billygoat-weed, chick weed and white weed. It used as medication in Asia, Africa, and South America (Vera, 1993; Okunade et al., 2002; Singh et al., 2013; Moore et al., 2020).

The essential oil of *A. conyzoides* has the potential antimicrobial, antiaflatoxigenic and antioxidant activity (Patil et al., 2010; Osho et al., 2011). *Zanthoxylum armatum* DC. (Rutaceae) is a shrub, and widely distributed in Asian region. The leaf, seed and fruit essential oil of *Z. armatum* has antibacterial, antifungal and anti-inflammatory activity (Mehta et al., 1981; Guo et al., 2010; Sati et al., 2011). *Mentha arvensis* L. belongs to the family Lamiaceae, native to temperate regions of Europe, western and central Asia, east to the Himalaya and eastern Siberia and North America. A lot of antimicrobial activity recorded of *M. arvensis*. The major bioactive compounds of *Mentha* are menthol, menthone, eugenol and pulegone (Luca et al., 2011; Moghtader, 2013; Akbar et al., 2014).

Herbal-based approaches come forward for development of new strategies to manage the plant diseases. A lot of plants essential oil used in the management of plant diseases and reduced the fungal as well as bacterial growth. Some plants like *Origanum vulgare*, *Cuminum cyminum*, *Eucalyptus citriodora*, *Thymus vulgaris*, *Cymbopogon citrates*, *Lavandula officinalis*, and *Zingiber officnalae* have essential oils that are reported for its antifungal activity (Lee et al., 2007; Cosic et al., 2010; Saroj et al., 2018; Raveau et al., 2020). In this study, *A. conyzoides*, *Z. armatum*, and *M. arvensis* essential oil used to the management of fungal pathogen *C. lunata*, cause leaf spot/leaf blight disease. The comparative antifungal activity of Menthol, Dementhol (DMO), and *M. arvensis* essential oil against *C. lunata* also studied in this paper.

MATERIAL AND METHODS

Plants and essential oils: The fresh *A. conyzoides* plant sample was collected in the plastic bag from the Lakhimpur, Pantnagar, and Kanpur, Uttar Pradesh, India. Whereas *Z. armatum*, and *M. arvensis* plants were sampled from the CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India. Sampled plants were taken quickly to laboratory and the fresh herbages were processed for essential oil extraction by the hydrodistillation method in the clevenger type apparatus (Maji et al., 2013). Extracted oils were purified and collected in to glass vial for further use.

In vitro screening of essential oils: Potato dextrose agar (PDA) plates have been prepared by adding different concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 mg/mL) of essential oil {(A. conizoides (code L1, P1 and K2)}at 40-45°C. To ensure the proper mixing of essential oil, 0.05% Tween-80 was added. A 6 mm disc of *C. lunata* was placed on the PDA filled Petri plates. All Petri-plates were incubated at 27±1°C. Plates without essential oil served as a control. The effect of essential oil on the growth of *C. lunata* was measured after five days of incubation. Minimum inhibitory concentration (MIC) was calculated using poison food technique (Grove and Moore, 1962; Knobloch et al., 1989; Nene et al., 1993). Percentage of the growth inhibition was calculated as follows:

$$\% \text{ of growth inhibition} = \frac{\text{Growth in control} - (\text{Growth in treatment})}{(\text{Growth in control})} \times 100$$

In vitro screening of chemical fungicides: By using the poison food technique, the fungicides Ridomil Gold and Bavistin are also tested for antagonistic activity against *C. lunata* in the concentration of 0.2, 0.4, 0.6, 0.8mg/mL. This study helps in the comparative study of chemical fungicide against selective essential oils.

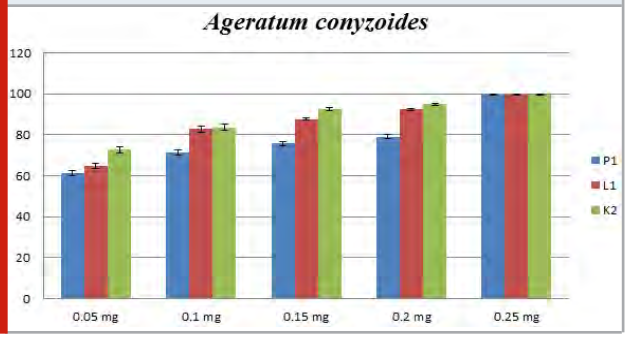
RESULTS AND DSICUSSION

Management of fungal diseases on plants using synthetic chemicals is more in practice. However, they possessed hazardous effect on plant, environment and human health. Thus, an alternate approach, employing of natural products viz Singh et al. (2019). Essential oil and chemical substances of plant origin, gained attention for the *in vitro* management of diseases caused by a plant pathogen. Here *Z. armatum*, *A. conyzoides*, and *M. arvensis* screen for the antifungal activity against the growth of *C. lunata* by Poison food technique.

Screening of essential oils against the growth of Curvularia lunata: Our results on in vitro screening showed that essential oils of *Ageratum conyzoides, Zanthoxylum armatum*, and *Mentha arvensis* proved highest antagonistic activity against the *Curvularia lunata* cause leaf spot/leaf blight diseases.

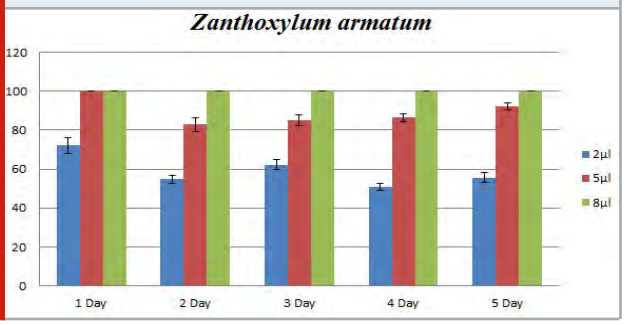
Ageratum conyzoides: Antifungal activity of *Ageratum conyzoides*, isolated from the different geographical location (Lakhimpur (L1), Pantnagar (P1), and Kanpur (K1) district of Uttar Pradesh) showed a similar type of growth inhibition. The concentration of 0.2mg has revealed a higher percentage (more than 90%) of inhibition as compared to others three concentrations of essential oils against *C. lunata* (Fig.1). The MIC was measured as 0.25 mg/mL, showed 100% of inhibition.

Figure 1: Effect of P1, L1 and K2 essential oils on phytopathogenic *C. lunata*. Percentage of growth inhibition measured using poison food technique. (P1, L1, and K2 shows that *Ageratum conyzoides* get from the different geographical location as Pantnagar, Lakhimpur and Kanpur respectively).



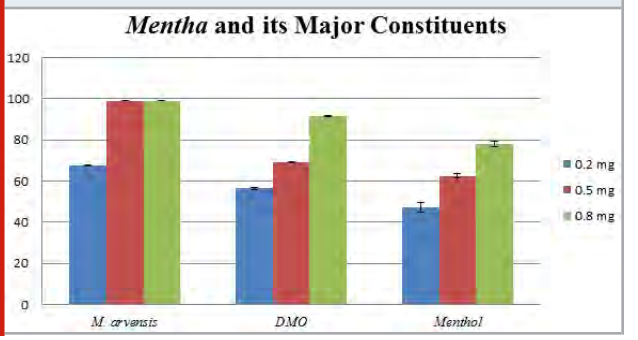
Zanthoxylum armatum: *Z. armatum* (8µl) showed 100% of inhibition of *C. lunata* by applying poison food technique while 5µl oil resulted in 80-100% of inhibition. Bar diagram revealed the percent of inhibition by applying 2, 5, and 8µl of essential oil continuously for five days (Fig.2).

Figure 2: Effect of *Z. armatum* essential oil on phytopathogenic *C. lunata*. Percentage of growth inhibition studied using poison food technique.



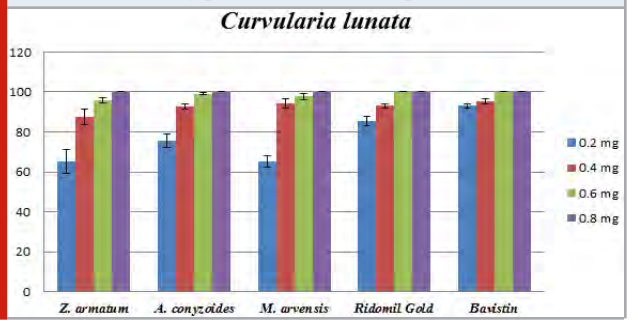
Mentha arvensis: Menthol is the major constituent of *M. arvensis* essential oil, discussed and proved its potential by various researchers as described in the review of literature section. Removal of menthol from *M. arvensis* essential oil, Dementhol or DMO remains present in the oil. Comparative analysis among menthol, DMO and *M. arvensis* oil; *M. arvensis* oils show greater antifungal activity against *C. lunata* as compared to menthol and DMO as denoted in the bar diagram (Fig.3).

Figure 3: Comparative effects of *M. arvensis* EO, DMO, and Menthol against phytopathogenic *C. lunata*. The mean of three independent experiments shows the percentage of growth inhibition using poison food technique.



Comparative study of Essential oil vs. specific fungicide: From above result, it shows that *Z. armatum* (8mg/mL), *A. conyzoides* (0.25mg/mL) and *M. arvensis* (0.8mg/mL) showed 100% of inhibition against *C. lunata* by applying poison food technique. Results in the case of selective chemical fungicides such as Ridomil Gold and Bavistin demonstrated 100% of inhibition when applied with a concentration of approx 0.5 mg/mL against *C. lunata*. Therefore, selective essential oils showed results to be at par as selected chemical fungicides (Fig.4).

Figure 4: Comparative effect of *Z. armatum, A. conyzoides, M. arvensis* oils, Reidomil Gold, and Bavistin on phytopathogenic *C. lunata*. Percentage of growth inhibition established using poison food technique.

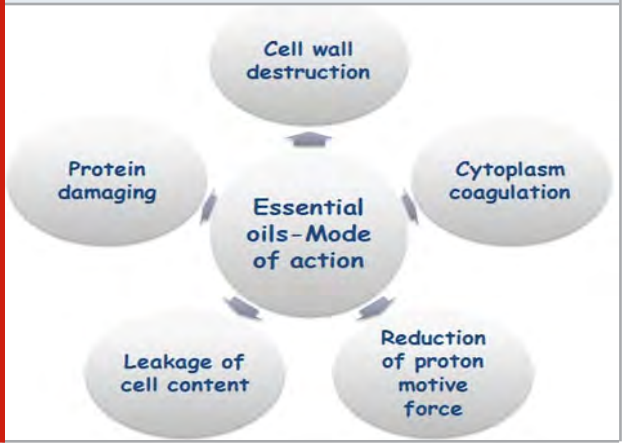


Many researchers have studied the antagonistic activity of essential oils and its compound against bacteria and fungus. Essential oils are the mixture of compounds so, it is most likely to show antimicrobial activity is not due to a single compound therefore it may be a synergistic/ synchronous effect (Skandamis et al., 2001; Carson et al., 2002).

Proposed Mechanism of fungal inhibition by essential oil: The antifungal activity of essential oils and their components is based on the feature of essential oils

that plays a critical role in antagonistic activity is hydrophobicity interaction with the cell membrane. It enables them to penetrate in the lipids of the cell membrane and mitochondria; disturbing the cell structure and causing cell lysis/cell bursting resulting into the leakage of ions and other cell contents (Knobloch et al., 1989; Sikkema et al., 1994; Oosterhave et al., 1995; Helander et al., 1998; cox et al., 2000; Lambert et al., 2001; Skandamis et al., 2001; Ultee et al., 2002; Carson et al., 2002; Pragadheesh et al., 2013). Figure 5 shows the mode of action of essential oils on the fungal cell.

Figure 5: Diagrammatic representation of essential oil actions on fungal cell



Expansion in population and food demand lead the development of new strategies for enhancing the agricultural crops resistant to any constraint. To avoid phytopathogenic diseases conventional pesticides applied by the farmers on the agricultural crop. However, conventional pesticides are cheap and lead to loss of nutrients in the soil and agricultural products as well (Singh et al., 2020). Thus, biological pesticides come in light, which is effective on plant diseases and sustain the quality of soil and plant products. Essential oil is the most effective biological pesticides used in the management of plant diseases. However chemical compositions of the essential oils are very complex because of the huge number of different groups of chemical constituents present in it. Thus, it is most likely that their antimicrobial activity is not due to one compound alone or by single mechanism but might be there are several targets in the cell (Skandamis et al., 2001; Carson et al., 2002; Raveau et al., 2020).

CONCLUSION

Fungi are the major causative organism of plant diseases on the MAPs as well as agricultural crops. The incidence of *C.lunata* fungi seems to be higher on the MAPs field shown in the recent years. Thus, the management of leaf spot/leaf blight diseases on sustainable basis should be necessary for save the economy of our country. In the present study, *M. arvensis* essential oil shows greater antifungal activity as compared to its major compound menthol and DMO via poison food technique. It means that there is a synergistic effect of menthol and another compound present in the oil to show greater antifungal activity while menthol or DMO show lesser activity. Along with *M. arvensis, A. conyzoides* and *Z. armatum* also exhibited significant pharmacological and biocidal activity.

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Asiatic Acid Attenuate Type 2 Diabetes Mellitus Induced Alterations in Acetylcholinesterase and Antioxidant System of Brain in Rats

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ABSTRACT

Diabetes is a metabolic disorder which manifests itself in an extensive diversity of complications including neurodegeneration. The treatment with antioxidants has been reported to produce beneficial effect in various types of neurodegenerative diseases. In the present study diabetes was induced by the administration of streptozotocin (STZ; 45 mg/kg body weight) and nicotinamide (NAD; 110 mg/kg body weight) to rats by intraperitoneally and hyperglycemia was confirmed by the elevated glucose levels (>250 mg/dL) in the blood. Then we investigated the effect of the administration of Asiatic acid (AA) is a triterpene and an active constituent found in large quantities in *Centella asiatica*, on biochemical parameters, acetylcholinesterase (AChE) activities, lipid peroxidation, enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic (glutathione) antioxidants in brain of normal (non diabetic) and STZ-NAD-induced diabetic rats. Changes in blood glucose, insulin, insulin resistance, lipid peroxidation, enzymatic and nonenzymatic antioxidants and activities of AChE were observed in normal control and experimental diabetic rats. Administration of AA (40 mg/kg body weight) for 45 days to STZ-NAD-induced diabetic rats showed a significant ($P<0.05$) reduction in plasma glucose, insulin resistance, and brain lipid peroxidation and AChE activity while brain enzymatic and non-enzymatic antioxidants levels are increased. The therapeutic effect of AA was compared with gliclazide, a well-known antioxidant and antihyperglycemic drug. AA supplementation significantly ameliorated all alterations induced by STZ-NAD in rats. Taken together, our study clearly depicts that AA, as a powerful antioxidant, prevents AChE, oxidative damage in the STZ-NAD-induced diabetic rats representing their possible therapeutics to be investigated in brain disorders associated with diabetes.

KEY WORDS: ANTIOXIDANTS, ASIATIC ACID, ACETYLCHOLINESTERASE, DIABETES MELLITUS.

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INTRODUCTION

Diabetes mellitus (DM) is probably highest growing metabolic syndrome in the globe and its increasing incidence puts a large burden on society and the public health sector. DM is spreading like an epidemic disease and India emerges to be the capital (Sathibabu et al., 2019). DM is characterized by the accumulation of glucose in the blood which causes hyperglycemia, often leads to various macrovascular and microvascular complications (Brahmanaidu et al., 2016; Uddandrao

et al., 2020). The neurological consequences of DM in the Central Nervous System (CNS) are now receiving greater attention. Diabetic Neuropathy (DNP) affects nearly 50% of diabetic patients with either type 1 or type 2 DM and involves both the peripheral and central nervous systems (Saravanan and Ponmurugan, 2013). During diabetic state, oxidative stress is caused by hyperglycemia that may be amplified an autocatalytic cycle of metabolic stress, tissue damage, and cell death leading to a simultaneous increase in free radical production and compromised inhibitory and scavenger mechanisms. Many studies proved that high levels of lipidperoxidation and low antioxidant defenses increase the vulnerability of the CNS to oxidative damage which causes alteration brain energy metabolism (Ramkumar et al., 2004; Saravanan and Ponmurugan, 2013.

The pathogenesis of DM is managed by insulin and oral administration of hypoglycemic medicines (Saravanan et al., 2009). Increasing of side effects and disease caused by drugs and chemotherapeutics agents provoke awareness toward natural products to minimize hazards like tissue and vascular complications caused by these drugs. Thus, new, relatively non-toxic, therapeutic representatives are required to treat hyperglycemia, which also would correct DNP (Saravanan and Ponmurugan, 2013. According to several in vitro and in vivo studies, in animals and human, dietary terpenoid compounds alter hyperglycemia, dyslipidemia and insulin resistance (IR), and improve oxidative stress and inflammation markers. In addition, they can inhibit the progression of many complications of DM involving retinopathy, nephropathy, neuropathy and cardiovascular disease (Moneim et al., 2017; Parim et al., 2019).

Asiatic acid (AA) is a triterpene found abundantly in *Centella asiatica* (L.), a perennial herbaceous creeper of the Apiaceae family, commonly used as the major constituent in a salad, juices and as ingredient in culinary dishes (Rameshreddy et al., 2018; Uddandrao et al., 2019). AA has an extensive array of biological functions including antihyperlipidemic, antihyperglycemic and anti-inflammatory activities (Swapna et al., 2019; Kalidhindi et al., 2020). However, no information pertaining to the anti-neurodegenerative effects of AA in the diabetic brain effects of is available. Thus, the present investigation aimed to assess the beneficial effects of AA against brain neurodegeneration in type 2 diabetic rats.

MATERIAL AND METHODS

Chemicals: AA (2α, 3β, 23-trihydroxyurs-12-en-28-oic acid was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All the reagents used in the experiments were analytical grade reagents of the highest purity.

Animals: Male Wistar rats were obtained from Department of Biochemistry, Muthyammal College of Arts and Science, Rasipuram, Tamil Nadu, India. The experimental rats were maintained under standard laboratory conditions (temperature: 22°C ± 2°C; humidity: 40%-60%) and were permitted food and water *adlibitum*. The rats, initially weighing 180–200g were divided into four groups of six each (n=6). All procedures involving laboratory animals were in accordance with the institutional animal ethical committee of Muthyammal College of Arts and Science (Approval No: IAEC/MCAS/05/2017).

Induction of DM: The overnight fasted rats were made diabetic by a single intraperitoneal injection of freshly prepared STZ (45 mg/kg BW) in citrate buffer (0.1 M, pH 4.5), 15 min after the intraperitoneal administration of Nicotinamide (110 mg/kg BW) in 0.9% normal saline. Hyperglycemia was confirmed by the elevated glucose levels (Above 250 mg/dL) in blood, determined at 72 h and then on day 7 after injection.

Experimental design: After the successful induction of experimental DM, the rats were divided into four groups each comprising a minimum of six rats.

Group 1: Normal control
Group 2: Diabetic control
Group 3: Diabetic rats orally treated with AA (40 mg/kg BW) in vehicle solution for 45 days through an intragastric tube (Swapna et al., 2019).
Group 4: Diabetic rats orally treated with Gliclazide (5 mg/kg BW) in vehicle solution for 45 days through an intragastric tube (Pulido et al., 1997).

Body weight and blood glucose level measurements were conducted periodically. At the end of the experiments, all the animals were anaesthetized using mild chloroform then decapitated. Brain structures were removed and separated into cerebral cortex and hippo campus. Blood samples were collected into tubes containing 2% sodium oxalate as an anticoagulant. The samples were centrifuged at 250 ×g for 5 min at 4°C, and then the plasma was immediately removed and stored at –20°C until analyzed. Blood glucose level was determined by using Span Diagnostic kit, Mumbai, India.

Determination of blood glucose, insulin and IR: Blood glucose level was estimated spectrophotometrically using reagent kit purchased from Spinreact Co. (Spain). Insulin levels in serum were estimated using specific ELISA kits (R&D Systems Inc., USA) according to the manufacturer's instructions. Insulin resistance was calculated by homeostasis model assessment of IR (HOMA-IR) as described by Rameshreddy et al. (2018).

Determination of antioxidants in brain: Brain tissues were weighed and rinsed in ice-cold saline. A 10% tissue homogenate was prepared using 0.025 M Tris-HCl buffer, pH 7.5. After centrifugation at 2000 rpm for 10 min, the clear supernatant was used for further biochemical assays. The supernatants thus obtained were used for the estimation of thiobarbituric acid substances (TBARS) (Fraga et al., 1988), assay of reduced glutathione (GSH) (Ellman, 1959), superoxide dismutase (SOD) (Kakkar et al., 1984), catalase (CAT) (Aebi, 1984), glutathione

peroxidase (GPx) by Paglia and valentine (1967) and glutathione-S-transferase (GST) activity was assayed by the method of Habig and Jackoby (1981).

Determination of Cholinergic dysfunction: Cholinergic dysfunction was assessed by measuring acetylcholinesterase (AChE) levels in cerebral cortex and hippo campus according to the method of Ellman et al. (1961).

Figure 1: Effect of AA on (A) body weight changes and (B) blood glucose in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.

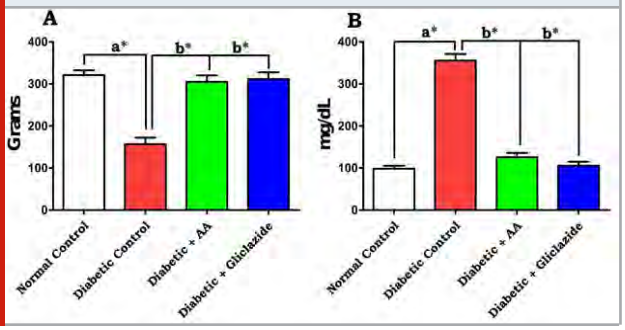
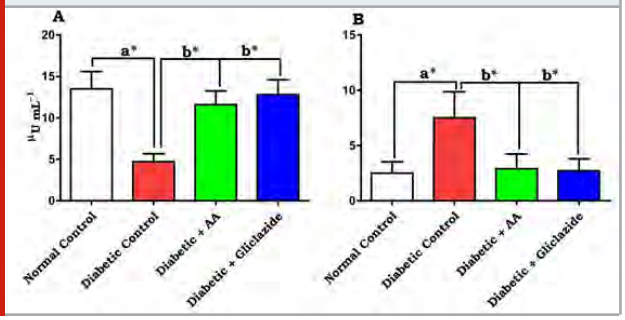


Figure 2: Effect of AA on (A) insulin and (B) IR in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.



Statistical analysis: All the results were expressed as the Mean ± S.D. (n=6). All the grouped data were statistically evaluated with graph pad prism software. Hypothesis testing methods included ANOVA followed by bonferroni multiple comparison test. Significance level at P<0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

In this study, DM was induced through STZ-NAD in rats. The mechanisms by which STZ-NAD brings about its diabetic state include selective devastation of pancreatic insulin secreting β-cells, which makes the cells less active (Pari and Srinivasan, 2010; Brahmanaidu et al., 2017) and leads to deprived glucose utilization by tissues. In the present study, STZ-NAD treatment to experimental animals confirmed the nature of DM as evidenced by decreasing body weight, insulin, and hyperglycemic condition and increased IR. This is in line with previous reports (Swapna et al., 2019). Figure 1 & 2 depicts the level of body weight changes (Fig. 1A), blood glucose (Fig. 1B), insulin (Fig. 2A) and IR (Fig. 2B) in control and experimental rats.

Figure 3A: Effect of AA on brain tissue lipid peroxidation in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.

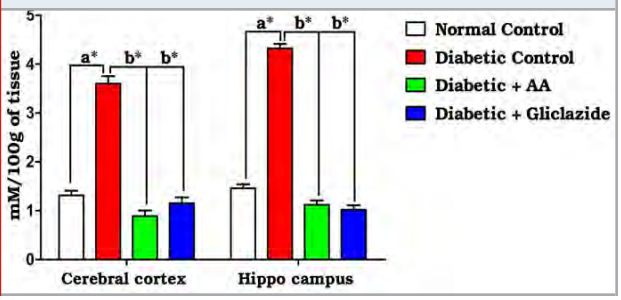


Figure 3B: Effect of AA on brain tissue hydroperoxides in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.

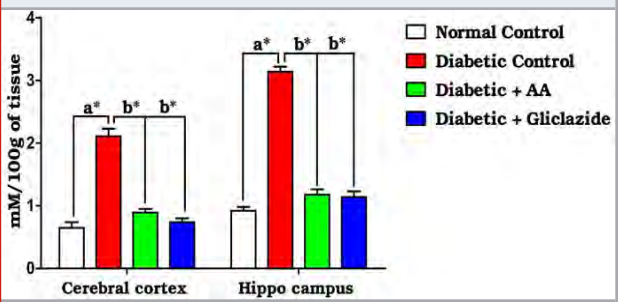
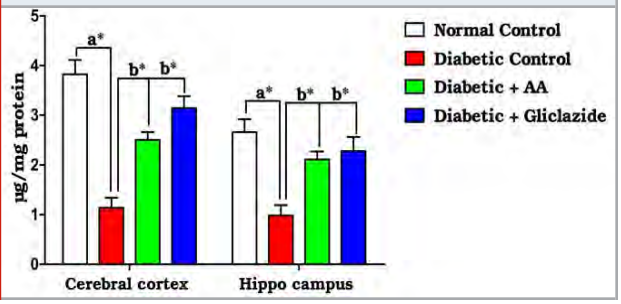


Figure 3C: Effect of AA on brain tissue GSH levels in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.



There was a significant (P<0.05) elevation in blood glucose level, IR and concomitant decreased level of body weight and insulin were noticed in STZ-NAD induced diabetic rats when compared with normal control. Administration of AA or gliclazide caused reduction in blood glucose, IR and increase in body weight and insulin

notably (P<0.05) when compared to the diabetic rats. AA produced hypoglycemic effect perhaps by enhancing the peripheral utilization of glucose, correcting the impaired hepatic glycolysis and limiting its gluconeogenic development parallel to insulin. In case of AA, the mechanism of reduction of blood glucose level might be due to increased peripheral uptake of glucose and increased sensitivity of insulin receptors (Saravanan et al., 2009; Uddandrao et al., 2019).

Figure 4A: Effect of AA on brain tissue SOD activity in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.

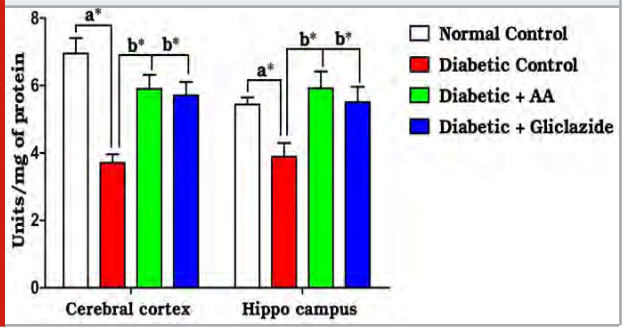
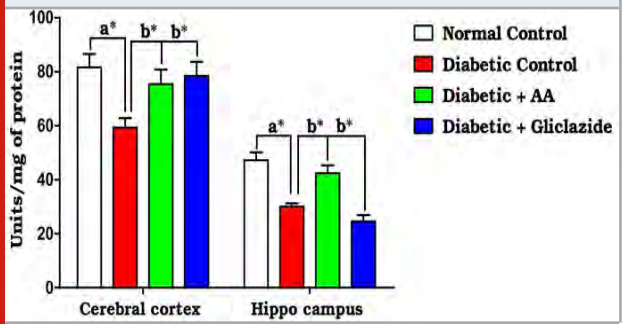


Figure 4B: Effect of AA on brain tissue CAT activity in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.



As summarized in figure 3A-C, there was a significant increase in the levels of brain TBARS (Fig. 3A) and hydroperoxides (Fig. 3B) in diabetic rats as compared to control rats. Treatment of diabetic rats with AA and gliclazide markedly ameliorated these altered parameters. On the contrary, brain GSH level (Fig. 3C) was obviously declined in diabetic rats and was significantly increased as a result of administration of AA and gliclazide. It is well known that chronic hyperglycemia causes an imbalance in the oxidative status of the nervous tissue which results damaging of brain through a peroxidative mechanism. This may be because the brain contains relatively high concentrations of easily peroxidizable fatty acids.

Vulnerability of brain induced by oxygen free radicals seems to be more due to the fact that the brain utilizes about one-fifth of the total oxygen demand of the body and it is not particularly enriched (Pari and Latha, 2004; Saravanan and Ponmurugan, 2013). Elevated metabolic rate, high lipid content and relative lack of antioxidant enzymes system might be the factors susceptible to oxidative damage of brain when compared to other organs. Thus, oxidative stress is considered the main player of many neurodegenerative diseases (Samarghandian et al., 2014; Uddandrao Sathibabu et al., 2017).

Figure 4C: Effect of AA on brain tissue GPx activity in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.

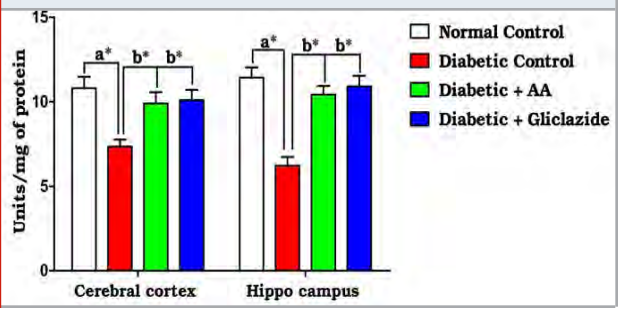
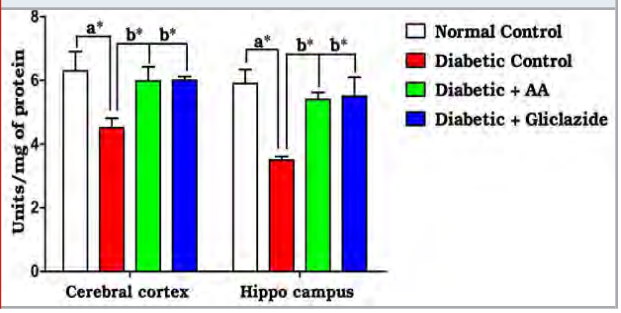


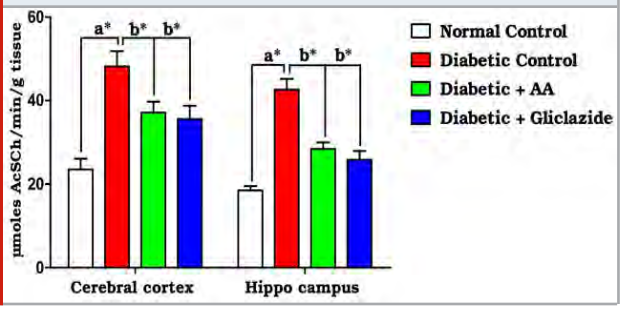
Figure 4D: Effect of AA on brain tissue GST activity in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.



Many studies on both diabetic humans and experimentally induced diabetic rats have revealed that hyperglycemia induces oxidative stress that may disturb brain function (Saravanan and Ponmurugan, 2013). In brain, increased levels of TBARS and hydroperoxides resulted from higher levels of LPO. Increased LPO under diabetic conditions can be due to increased oxidative stress in the cell as a result of depletion of antioxidant scavenger systems (Raza et al., 2015). Figure 4A-D indicates the level of the activities of antioxidant enzymes including SOD (Fig. 4A), CAT (Fig. 4B), GPx (Fig. 4C) and GST (Fig. 4D). There was a significant decrease in the activities of antioxidant enzymes in brain of diabetic rats as compared to control rats. Oral administration of AA and gliclazide potentially improved these altered activities.

Based on the above-mentioned data, AA has powerful free radical scavenging and antioxidant activities in the diabetic brain. More specifically, it has been shown that hippocampus of STZ-induced diabetic rats has increased oxidative stress and impaired antioxidant defense systems (Samarghandian et al., 2014). In the present study, a significant increment of TBARS and hydroperoxides and concomitant decrease in the activities of antioxidant enzymes (SOD, CAT, GPx and GST) as well as GSH content were observed in the brain of diabetic rats, which is brought back to normal by AA or gliclazide treatment. This might be due to antioxidant nature of AA that counteract the free radical generation during diabetes (Swapna et al., 2019).

Figure 5: Effect of AA on brain AChE activities in control and experimental animals. Values are expressed as mean±S.D, n=6, [a]Significantly different from normal control, [b]Significantly different from diabetic control, *P<0.05.



AChE, a significant biological component of the membrane, contributed to its integrity and changed in permeability occurring during synaptic transmission and conduction. Many studies have demonstrated that learning and memory can be modified by drugs affecting the central cholinergic system. Cholinergic transmission is terminated mainly by acetylcholine hydrolysis via the enzyme AchE. One of the hallmarks of this mechanism is impaired cholinergic neurotransmission (Cunnane et al., 2011; Balbaa et al., 2017). The activities of tissue AChE in normal and experimental rats are shown in figure 5.

The brain of diabetic rats showed significant increase (P<0.05) in tissue AChE activity. Significant prevention in the activities of tissue AChE was observed in diabetic rats after supplementing AA or gliclazide. Beheshti et al (2016) reported that the metabolism of glucose and impaired insulin signaling in brain is associated with acetylcholine synthesis and the reduced glucose levels in the brain may have a negative effect on cognitive function.

During the diabetic state, the alterations in the lipid membrane could be a key factor in the modification of the conformational state of the AChE molecule and would explain the changes in the activity of this enzyme. It is due to the increased level of free radical formation which promoted increased LPO, having as major consequence oxidative deterioration of the cellular membranes. Thus, free radical scavengers and antioxidants have been shown to prevent neurodegeneration (Mehta and Banerjee, 2017). Reduced GSH has been demonstrated to be an important marker for oxidative stress in the cortex and hippocampus (Pocernich et al., 2012; Salama et al., 2016).

In the present study, decreased GSH level in cortico-hippocampal lysates of STZ-NAD animals was observed which ultimately leads to increase in AChE activities in the different brain regions. AA treated experimental animals showed a decrease in AchE and increase in GSH levels, which was comparable to control animals. Earlier, Tuzcu and Baydas (2006) and Kuhad et al., (2008) reported that the antioxidant therapy may decrease the AChE activity induced by the diabetic state. In line with this, AA, an antioxidant may be responsible for preventing cholinergic dysfunction in diabetic rats (Rameshreddy et al., 2018; Swapna et al., 2019).

CONCLUSION

In conclusion, administration of AA rise antioxidant enzymes to a relevant extent in STZ-NAD induced diabetic animals and to retard brain AChE associated with hyperglycaemic development experimentally. From this study, altering the antioxidant enzymes will be a promising new pharmacological approach to treat brain disorders associated with DM. Further investigation into the neuroprotective potential and mechanisms of AA is required to determine, whether it can be an effective cure for cognitive impairment.

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Conflict of interest: The authors declare no conflict of interest.

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Spread of Toxoplasmosis in Humans and Animals in the Tyumen Region

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ABSTRACT

The objective of the research was to study the spread of toxoplasmosis in people and animals in the Tyumen region. Studies on the diagnosis of toxoplasmosis in humans were carried out at the Federal Budgetary Institution of Science "Tyumen Scientific and Research Institute of Territorial Infectious Pathology" of Rospotrebnadzor (Tyumen) in 2000-2015. Diagnosis of toxoplasmosis in animals was carried out in the Tyumen Regional Veterinary Laboratory in 2011-2016. The disease incidence among people in the region was analyzed using the results of immunological studies of various age groups of the population. To diagnose toxoplasmosis in animals, parasitological and immunological methods of diagnosis were used. It was established that toxoplasmosis is a widely common disease among people living in the Tyumen region. The maximum number of positive reactions to toxoplasmosis was recorded in 2002, 2003, 2008, and from 2012 to 2016, when the level of seropositive reactions was 26.2; 20.2; 12.26; 17.08; 11.36; and 10.59% of people examined for toxoplasmosis. In 2014, the highest seropositivity rates for *T.gondii* were 250.35 and 151.09 per 100 people, respectively, in Abatsky and Omutinsky districts of the Tyumen region. Among children, the invasion was recorded at the age of over one year with the maximum seropositive level - 4.36% in 2012. Among dogs and cats examined for the presence of antibodies to *T.gondii*, the maximum level of seropositivity was found in 2013 and 2016. These indicators were at the level of 15.4 and 15.0%, respectively. During the examination of seropositive cats, the release of oocysts of *T.gondii* was found in only 0.42%.

KEY WORDS: TOXOPLASMOSIS, SEROPOSITIVITY, TYUMEN REGION, DOGS, CATS.

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INTRODUCTION

One of the important problems of medicine and veterinary medicine that has serious socio-economic importance is toxoplasmosis. Special attention to this disease is justified by its consequences, which are most important for a person, and especially to a developing fetus when a pregnant woman is infected during pregnancy. The widespread occurrence of toxoplasmosis among animals, especially free-living and wild, does not allow for its control and affects more and more susceptible

organisms(Gao, et al., 2020). For the first time, the causative agent of toxoplasmosis - *Toxoplasma gondii*, was mentioned in 1908 in Tunisia and Brazil in Gundi (a species of rodents) and rabbits, respectively (Jitender Prakask, 2016; Jones and Dubey, 2012; Pappas et al., 2009; Lehmann et al., 2006; Nasiru Wana et al., 2020).

The first case of congenital toxoplasmosis was diagnosed in 1923 (Chandramathi and Nissapatorn, 2019; Petersen et al., 2010). To date, the number of persons infected with toxoplasma exceeds 1.5 billion people, and the prevalence of toxoplasma in different areas varies from 14 to 90%, averaging at least 35% (Fiedler et al., 1999; Hofhuis et al., 2011; Ben-Harari and Connolly, 2019; Kijlstra and Jongert, 2008). The minimal prevalence of the population is noted in the Nordic countries - 14%, low, in New Zealand, Great Britain and Australia- 25%, the average level - 35-50% in many countries of Asia, Africa, America, and Europe (Fiedler et al., 1999; Glazunova et al., 2018; Ben-Harari and Connolly, 2019; Jones et al., 2009; Lehmann et al., 2006).

This is evidenced, in particular, by the fact that a high percentage (60–90%) of individuals with antibodies to toxoplasma (Robert-Gangneux and Dardé, 2012; Lehmann et al., 2006) was detected among the population of several countries in Asia and Western Europe. Annual rates of seroconversion in countries with a high prevalence of the population are more than 3%, in the "toxoplasmosis-safe" countries of Northern Europe and in the "relatively safe" UK and USA, this indicator is less than 1% (Chandramathi and Nissapatorn, 2019). But even if looking at the minimum indicator, 0.6% of the US population who annually suffer the acute phase of invasion amount to about 1.5 million cases of the disease, and approximately 15% of them are clinically significant (Mead et al., 2000). In different territories of Russia, invasion of the population (according to quite incomplete data) is on average 30-35% (Aliyu et al., 2020; Jitender Prakask, 2016).

Due to the lack of awareness of the population each year, hundreds of cats are euthanized or thrown into the streets, aggravating the problem of neglected animals. Home-living animals who have never encountered *T. gondii*, risk being infected, as a result of which they become subjects of the excretion of Toxoplasma cysts, infecting children's playgrounds, especially sandboxes, municipal water bodies, and lawns. A vicious circle occurs. The spread of toxoplasmosis in populated areas is due to the lack of a systematic approach to the regulation of the number of homeless animals (Gao, et al., 2020). In 2012-2018 in Tyumen, 9623 dogs were taken from the streets of the city, most of which were returned back without prior examination for toxoplasmosis and no action taken depending on the situation.

Thus, a systematic approach to the problem of toxoplasmosis is required, where human doctors, veterinarians, breeders, and owners of animal nurseries, epidemiological control services, and every person would work to educate the population, early diagnose, prevent the disease, interrupt the development cycle of the pathogen and eliminate it from the environment.

Given the significance of the problem and the lack of current knowledge about the distribution of toxoplasmosis in the Tyumen region, we set a goal to study the spread of toxoplasmosis in humans and domestic animals in the Tyumen region. In other words, in the current study, it was tried to study and analyze the spread of toxoplasmosis in people and animals in the Tyumen region. Studies on the diagnosis of toxoplasmosis in humans were carried out at the Federal Budgetary Institution of Science "Tyumen Scientific and Research Institute of Territorial Infectious Pathology" of Rospotrebnadzor (Tyumen) in 2000-2015.

MATERIAL AND METHODS

Studies on the diagnosis of toxoplasmosis in humans were carried out at the Federal Budgetary Institution of Science "Tyumen Scientific and Research Institute of Territorial Infectious Pathology" of Rospotrebnadzor (Tyumen) in 2000-2015. Diagnosis of toxoplasmosis in animals was carried out in the Tyumen Regional Veterinary Laboratory in 2011-2016. The disease incidence among people in the region was analyzed using the results of immunological studies of various age groups of the population. To diagnose toxoplasmosis in animals, parasitological and immunological methods of diagnosis were used. The parasitological methods were the microscopy of smears of the affected organs and stool tests. 723 stool tests were conducted. The immune-chromatographic analysis was performed using the rapid test system (Quicking Biotech Co., Ltd.); the immunological methods were enzyme immunoassay used to detect IgG and IgM (Hellmann et al., 2018; Sierra et al., 2020).

Immunology: The immune system is a complex system of structures and processes that have evolved to protect us from the disease. The function of these components is divided into two parts. The first part of which responds non-specifically to all microbes in the same way, which is called the innate immune response. The second part is the responses that specifically respond to a specific microbe known as acquired immunity. Immunology is the study of the immune system and is a very important branch of medical science and biology. Our immune system protects us from various infections. If the immune system does not work this way, it can lead to diseases such as autoimmune diseases, allergies and cancer.

Toxoplasmosis: Toxoplasmosis is one of the parasitic diseases of humans and animals is toxoplasmosis, the initial infection of which is asymptomatic. The severe and acute type of disease is accompanied by fever and enlargement of the lymph nodes. Symptoms of a rare form of the disease that is rarely seen include brain symptoms, pneumonia, generalized muscle disease, and death. The disease is caused by *Toxoplasma gondii*, an intracellular protozoan that is transmitted through fecal-oral feces from cat feces that contain infected oocytes or parasite eggs. The causative agent may occur through

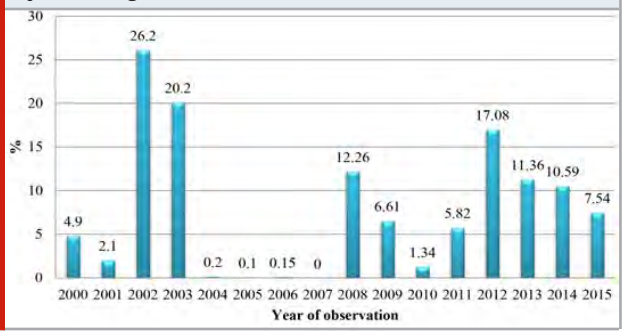
eating uncooked meat, transfusing blood or organ transplants, or through the placenta into the fetus despite an acute infection in a pregnant woman.

Chromatography: Chromatography is an analytical method commonly used to separate the components of a chemical mixture. As a result, with the "chromatography" method, it is possible to analyze the components of the mixtures well. There are several methods of chromatography, including liquid chromatography, gas chromatography, ion-exchange chromatography, and affinity chromatography, all of which have the same basis.

RESULTS AND DISCUSSION

We established that the epizootological situation of toxoplasmosis among people is constantly changing and the number of seropositive reactions varies from 0 to 26.2% of those examined. Thus, no cases of toxoplasmosis among people were recorded in 2007; this period was preceded by an intense decline in positive results (Figure 1). The maximum number of positive reactions to toxoplasmosis was recorded in 2002, 2003, 2008, and from 2012 to 2016, when the level of seropositive reactions was 26.2; 20.2; 12.26; 17.08; 11.36; and 10.59% of people examined for toxoplasmosis.

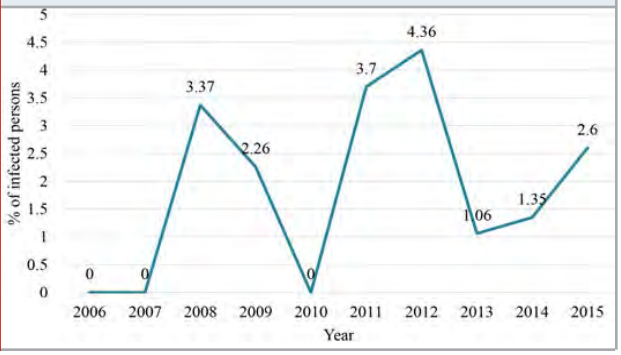
Figure 1: The level of the infected population of the Tyumen region, share, % (2000-2015)



The frequency of detection of antibodies to *T.gondii* varied depending on the residential area of the population. The population of Abatsky and Omutinsky districts turned out to be the most infected. In 2014, the rate of infection of the population with toxoplasmosis per 100 thousand people reached its maximum of 250.35 and 151.09, respectively. In 2015, the situation remained virtually unchanged, and the level of invasion in the same areas was 229.4 and 137.5. At the same time, in the neighbouring areas, the infection rate was significantly lower and ranged from 0 to 27.38.

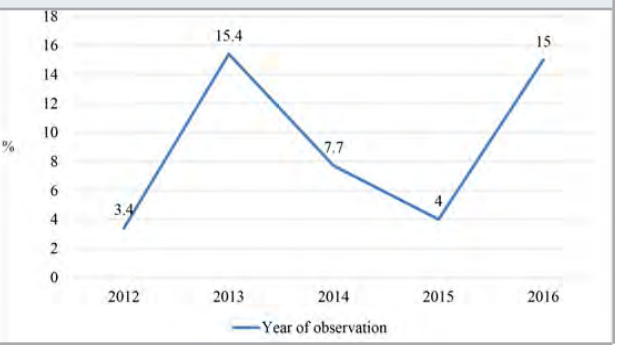
The situation of *T.gondii* invasion among urban residents of the Tyumen region has also been clarified. The results of research in the cities of Tyumen, Ishim, and Tobolsk were analyzed. Residents of the regional centre were found to be the most infected population, while the maximum number of seropositive people in 2012 was 32.34 per 100 thousand population. In other cities, the level of the seropositive population did not exceed 4.18. Moreover, in 2006-2015, the *T.gondii* invasion rate was studied among children aged 0 to 17 years. It was revealed that only one child under one year had antibodies to the specified pathogen.

Figure 2: The *T.gondii* invasion rate among children aged 0-17 years in the Tyumen region (2006-2015)



The peaks of the invasion were in 2008, 2011, and 2012, when the detection rate of antibodies to *T.gondii* was 3.37; 3.7; and 4.36%. We should note that the level of seropositive children under 14 is higher. In 2008 it was 4.31, in 2011 - 3.96, and the maximum indicator among children was found in 2012 - 5.13. Considering that animals are the main spreaders of invasion, we found out the prevalence of toxoplasmosis in domestic animals. It was found that in five studies (2012-2016) the level of seropositive animals varied (Figure 3). Thus, in 2012 and 2015, the level of seropositivity among dogs and cats was 3.4 and 4.0%, respectively. Whereas in 2013 and 2016, these indicators were 15.4 and 15.0%, respectively.

Figure 3: The share of *T.gondii* seropositive dogs and cats in the Tyumen region (2012-2016)

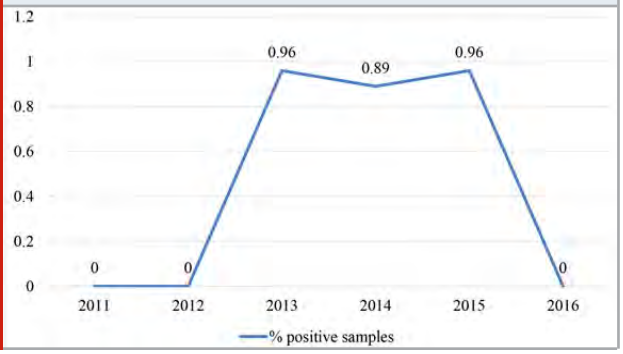


In parallel with the immunological method of diagnosis, 723 stool test of cats were conducted to detect oocysts of toxoplasma (Figure 4). We found that *T.gondii* oocysts are extremely rare in seropositive cats. In 2013-2015, only three samples contained *T.gondii* oocysts.

It is considered that 25-30% of the world's population is infected with *T.gondii* (Robert-Gangneux and Dardé, 2012). The level of the seropositive population in the Tyumen region is within the average statistical indicators for Russia. Thus, in the Russian Federation,

25% of the population surveyed in Moscow and 32% of the population surveyed in the Oryol region are seropositive to *T.gondii* (Aliyu et al., 2020 Prakask, 2016). In Krasnoyarsk Krai, 28.6% were seropositive the level of seropositivity among the surveyed pregnant women in the Belgorod region was 23.6±1.4% (Prakask, 2016). The results of immunological studies among people from the neighbouring Omsk region, where the growth of toxoplasmosis was 2.0 times higher in 1992-2006, coincide. The Tyumen region also had the highest rates in 2002 and 2003 - 26.2 and 20.2%, respectively, while in 2004-2007, there was the lowest seropositivity recorded - 0-0.2% (Prakask, 2016 Gao, et al., 2020).

Figure 4: Oocysts in *T.gondii* seropositive cats (2011-2016)



There is evidence that the level of seropositivity to *T.gondii* depends on the socio-economic situation. Thus, the study of the epidemiological situation in Brazil found that antibodies against toxoplasma are found in 84, 62, and 23% of the population of the low, medium, and high class, respectively (Aliyu et al., 2020; Robert-Gangneux and Dardé, 2012; Schlüter et al., 2014). In the Russian Federation, 83.5% of the children surveyed in the Omsk region, who were seropositive for toxoplasmosis, were from single-parent large families with poor sanitary and living conditions (Jones et al., 2018 Ben-Harari and Connolly, 2019). The highest prevalence of toxoplasmosis (41.0%) was observed in children from rural areas on the background of tuberculosis infection.

Our results also confirm the assumption of the significance of social status and culture on the risk of *T.gondii* invasion. In the Tyumen region, the population living in rural areas is more seropositive, which may be due to the sanitary and hygiene offences, as well as a large number of homeless and free-living cats in the private sector. Considering the low level of release of *T.gondii* oocysts from seropositive domestic animals (0.42%), the probability of invasion of people eating semi-raw meat is also high (Glazunova et al., 2018; Jones and Dubey, 2012; Lindsay et al., 2002; Kijlstra and Jongert, 2008; Zulpo et al., 2018, Wana et al. 2020).

CONCLUSION

Analysis of the results allows us to conclude that toxoplasmosis is widespread among people living in the Tyumen region. The maximum number of positive reactions to toxoplasmosis was recorded in 2002, 2003, 2008, and from 2012 to 2016, when the level of seropositive reactions was 26.2; 20.2; 12.26; 17.08; 11.36; and 10.59% of people examined for toxoplasmosis. In 2014, the highest seropositivity rates for *T.gondii*were 250.35 and 151.09 per 100 people, respectively, in Abatsky and Omutinsky districts of the Tyumen region. As for urban residents of the region, toxoplasmosis is common among residents of the regional centre, with 32.34 seropositive people per 100 thousand population found in 2012. Among children, the invasion was recorded at the age of over one year with the maximum seropositive level - 4.36% in 2012. Among dogs and cats examined for the presence of antibodies to *T.gondii*, the maximum level of seropositivity was found in 2013 and 2016. These indicators were at the level of 15.4 and 15.0%, respectively. During the examination of seropositive cats, the release of oocysts of *T.gondii* was found in only 0.42%.

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Trend Analysis of Technology Sector FDI in India from Top Five Investing Countries

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ABSTRACT

This paper has attempted to analyse the patterns of FDI in the technology sector from the top five investing countries in India. In recent years, India's information technology industry has drawn a considerable amount of foreign direct investment. Investments are being made in four main sectors of the Indian information technology industry: online firms, IT services, IT services, and software merchandise. It is well known that FDI will complement local development activities in many ways, including improving export competitiveness; creating employment and reinforcing the skills base; enhancing technological capabilities; and rising financial capital development. In India, FDI has allowed a certain degree of financial stability to be achieved; development and growth to sustain and succeed in the global economy. Efforts made by the Indian government are also studied to attract FDI in the science and technology sector. According to official statistics, foreign direct investment (FDI) in India grew by 13 percent to a record of $49.97 billion in the financial years 2019-20. With investments of $14.67 billion, Singapore emerged as India's largest source of FDI during the last fiscal year. Mauritius ($8.24 billion), the Netherlands ($6.5 billion), the United States ($4.22 billion), the Caiman Islands ($3.7 billion), Japan ($3.22 billion), and France ($1.89 billion) followed. FDI in the IT sector accounted for 10% of total FDI in India during 2000-2019.

KEY WORDS: TECHNOLOGY SECTOR, FOREIGN DIRECT INVESTMENT, ECONOMIC GROWTH, INVESTING COUNTRIES, TREND ANALYSIS.

INTRODUCTION

India is the largest democracy in the world and is the fifth largest purchasing power parity economy in the world. India provides huge investment opportunities with its ample labor supply, unexplored markets, and adequate natural resources. This is the fastest-growing trillion-dollar economy in the world and has been the fifth largest economy in 2019 to surpass the UK and France, with a nominal GDP of $2.94 trillion. The nation ranks third when contrasting GDP to $11.33 trillion in terms of purchasing power parity. According to the IMF, India's growth rate is projected to rise from 7.3 percent in 2018 to 7.5 percent in 2019 as the currency exchange policy drags and the implementation of goods and services tax fade.

Since India is a developing country, capital is small, and socio-economic issues such as health, poverty, research and development, obsolescence of technology, education, global competition are many. The role of FDI in the host country's overall development is multidimensional. It helps to bring about the creation of foreign capital resources without debt, capacity enhancement, new jobs, spillovers, and allocative performance effects. FDI also promotes foreign exchange, information, expertise

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and technology transfer. The Government of India has therefore approved 100% FDI under the automatic approval route in the electronic hardware sector and the software development sector. In filling the gap between domestic saving and spending overall capital growth, FDI plays a major role, (Bhave, 2020).

The flagship 'Make in India' campaign of the Union government, launched in 2014, is led by the ideals of competitive federalism. It aims to leverage the capacity of the human, financial, physical, social and natural capital base of India and is focused on the premise that the economy can reap the benefits of FDI inflows by transforming India into a global manufacturing hub, (Saha & Bhowmick, 2020). Through defining the "rules of the game," public policy plays an important role in encouraging innovation. These include the rule of law, intellectual rights, patent protections, contracts, free trade policies, freedom to travel, numerous investment incentives, and light-touch regulations and regulatory regimes, (Nguyen, et.al. 2020). In this paper, an attempt has been made to study FDI inflows in the information technology sector and from the top five investing countries in India.

Review of Literature: Deivamani et al. (2018) have analysed the determinants of FDI, sources of FDI inflows in India during the financial year 2016-17. As per the study, the service sector and construction and development sector inflow of FDI attained sustainable economic growth and development through the creation of jobs in India. So to make the Indian economy a developed economy, the Government must proceed to elicit FDI inflows in India.. Murugesan (2016) have investigated the trend and growth of FDI inflows in the past liberalization period and the sector-wise FDI inflows received in India. This study has examined a significant effect of FDI on the economic growth and sector-wise FDI inflows received in India from 2006 to 2013. FDI inflows can be utilized as a means of improving domestic production, exports and saving through the equitable distribution FDI inflows can be used to raise the output, Productivity and trade at a sectoral level of the Indian economy.

Earlier Graeme (2015) had examined the determinants of FDI using a large sample of both developed and developing countries. The study finds that variables like size and scale of economic activity in the host country are the most significant variables in explaining FDI flows, while variables such as economic freedom, tax incentives and human capital are not at all significant. Roy (2012) studied the causal relationship between foreign direct investment (FDI) and economic growth for selected Asian economies covering the period from 1981-2008. It has been observed that for countries like China, India, Pakistan, Sri Lanka, Indonesia, Philippines and Singapore, the direction of causality runs from economic growth to FDI and not the other way round. However, for Malaysia, there is no causality between FDI and GDP. Only in the case of Thailand, a bidirectional causal relationship exists. Malaysia confirmed Granger neutrality whereas for Thailand bi-directional relationship exists. Countries such as China and India can compete for higher growth potentials.

Objectives: To analyse the trends of FDI inflow from top five investing countries and to Study inflow in the technology sector from these countries.

Research Methodology: Trend analysis Regression has been applied by using the model as:

$$\text{Log FDI}=c+\beta\,(t)+u$$

b) Exponential Growth rate is studied.

$$Y= abt$$

Table 1. Trend Analysis of Foreign Direct Investment Inflow in IT Sector from January 2000 to December 2018

Year	IT Sector	Total FDI	% Share of Total Inflows	% Growth as Per Preceding Year
2000	12008.32	104411	11.5	
2001	20566.93	160711	12.8	71.27
2002	31908.64	161345	19.8	55.15
2003	13550.09	95640	14.2	-57.53
2004	31,030.11	147814	21.0	129.00
2005	42,066.76	192707	21.8	35.57
2006	87,492.90	503573	17.4	107.99
2007	102,148.54	654950	15.6	16.75
2008	78,102.50	1595295	4.9	-23.54
2009	32,124.70	1309799	2.5	-58.87
2010	45,347.95	960150	4.7	41.16
2011	31,350.97	1599349	2.0	-30.87
2012	34,374.34	1215914	2.8	9.64
2013	36,598.31	1294825	2.8	6.47
2014	95,622.02	1753134	5.5	161.27
2015	42,537.03	2525614	16.8	344.85
2016	16,196.91	3116439.70	5.20	-
2017	45,701.84	2827679.50	16.05	
2018	41,733.18	2906952.30	14.13	-
Total	840462.04	23126303		
ECGR	9.42			
Constant		38620.13		
Reg. coefficient		0.033		
R-square		0.0942		
F-value		1.769		
P-value		.020		

Source: Various newsletters from 2000 to December 2018

Data Analysis: FDI IN INFORMATION TECHNOLOGY SECTOR In India's economy, the information technology (IT) sector has grown as a big success story. In the last

decade, the Government of India's liberalised policy reforms has pushed the Indian IT industry on to a road of growth and prosperity. In today's economies of the world, electronics and information technology are proving to be the growth driver. A comparatively new entrant in India's export horizon, the Electronics Hardware and Computer Software Services industry has emerged as a leader among all industries and has been trading consistently on a fast growth path in recent years. The industry's wise break-up of cumulative FDI inflow shows that after construction growth, computer software and hardware were placed third. Trend analysis reveals that gross total inflow grew at an annual exponential compound growth rate of 11.8 percent from Rs. 31,030.11 million in a year, 2004 to Rs. 41,733.18 million in the year, 2018. Its share of total FDI inflow decreased from 21 percent in 2004 to 2 percent in 2011. Thereafter, FDI gradually increased in this sector to Rs. 41,733.18 million (14.18 percent in 2004).

Table 2. Regions Wise FDI Inflows in Computer Software and Hardware from January 2000 to December 2019

S.No.	Regions	Amount	% age Share of FDI
1.	Bangalore	10,395.95	25.18
2.	New Delhi	9553.49	23.14
3.	Mumbai	9,208.78	22.30
4.	Chennai	2,071.92	5.02
5.	Hyderabad	1755.10	4.25
Total	32,985	79.89	

Source: SIA Annual Newsletter Issue 2019

Table 3: Country-Wise Equity FDI Inflows in Computer Software and Hardware from January 2000 to December 2019

S.No.	Countries	Amount of FDI	%age Share of FDI
1.	Mauritius	12,839.73	29.44
2.	Singapore	11,390.26	26.12
3.	U.S.A	5,622.22	12.89
4.	Netherlands	4,240.01	9.72
5.	Cayman Islands	2148.08	4.93
Total	36,240.30	83.10	

Source: SIA Annual Newsletter Issue 2019

Region-Wise FDI in Computer Software and Hardware Sector: Table 2 displays the area-wise break-up of FDI inflows from January 2000 to December 2019. With 80% FDI, Mumbai, New Delhi, Bangalore, Chennai and Hyderabad are the major FDI inflow centers in the IT market. In computer software and hardware, Bangalore led with 25.18 percent of total FDI inflows.

Country Wise FDI in the Information Technology Sector: Thus from January 2000 to December 2019, the country-wise break-up of total FDI inflows in the IT sector was given in Table 3. Mauritius, the United States, Singapore, the Netherlands and the Cayman Island together accounted for around 83.10 percent of FDI inflows in this sector. With 29.44 percent, Mauritius ranked at the top.

Table 4. Trend Analysis of Foreign Direct Investment from Mauritius from January 2008 to December 2018

Year	FDI from Mauritius	Total FDI Inflows	Growth of % Inflow	% Share of Out of Total
2008	598586.46	1595295.00	-	37.52
2009	560128.88	1309799.00	-6.42	42.76
2010	329399.98	960150.00	-41.19	34.31
2011	437791.67	1599349.00	32.91	27.37
2012	491418.84	1215914.00	12.25	40.42
2013	331290.51	1294825.00	-32.58	25.59
2014	429456.74	1753134.00	29.63	24.50
2015	590308.74	2525614.00	37.45	23.37
2016	1012330.1	3116439.70	71.49	32.48
2017	1053377.4	2827679.50	4.150	37.25
2018	593461.6	2906952.30	-43.700	20.415
Total	5834089.32	21105152		
ECGR	28.5			
Trends				
Constant value		366001.01		
Reg. coefficient		0.173		
R-square		0.285		
F-value		42.67		
P-value		.000		

Source: Various newsletters from 2000 to December 2018

Fdi From Mauritius: The Double Taxation Avoidance Agreement (DTAA) was signed by the Indian government and Mauritius in 1982. As a consequence, the maximum FDI comes to India via Mauritius. A distinctive feature of the double tax avoidance treaty concluded in August 1982 in Mauritius provides some indication as to why Mauritius remains India's chosen route for investment. The treaty stated that capital gains made on the sale of shares of Indian companies by investors in Mauritius would only be taxed in Mauritius and not in India. However, the treaty survived for the first 10 years only on paper, as foreign institutional investors were only allowed to invest in Indian stock markets. This changed in 1992 when foreign institutional investors (FIIs) were also allowed to enter India. Mauritius immediately took advantage of this move. Under the Business Activities

Act, multinational companies have been authorised to register for foreign investment in the island country.

The FDI inflow from Mauritius, however, remained important even though its share of FDI inflows declined, remaining one of India's largest sources of FDI. Based on the time factor with F value 42.67, the value of R square explains the 28.5 percent difference in FDI inflow, which is important at a 1 percent significance level. It shows a large fluctuation in Mauritius' FDI inflow. To summarize, in India, FDI inflows from Mauritius still dominate. It was limited to a few sectors of the Indian economy and was concentrated in the country's economic and industrial growth regions.

Table 5. Trend Analysis of Foreign Direct Investment from Singapore from January 2008 to December 2018

Year	FDI from Singapore	Total FDI Inflows	Growth as Per Preceding	% Share of Total
2008	157,758.64	1595295.00	170.57	9.89
2009	148,262.44	1309799.00	-6.02	11.32
2010	96,757.80	960150.00	-34.74	10.08
2011	195,969.66	1599349.00	102.54	12.25
2012	152,421.76	1215914.00	-22.22	12.54
2013	222,116.31	1294825.00	45.72	17.15
2014	432,861.05	1753134.00	94.88	24.69
2015	862,990.95	2525614.00	99.37	34.17
2016	659,735.80	3116439.70	-23.55	21.17
2017	701,090.10	2827679.50	6.27	24.79
2018	1,086,273.00	2906952.30	54.94	37.37
Total	4,716,237.51	21105152		
ECGR	22.7			
Constant value		78975.45		
Reg. coefficient		0.227		
R-square .785				
F-value 29.29				
P-value .000				

Source: Various newsletters from 2000 to December 2018

Fdi From Singapore: Singapore was put second after Mauritius in the country-wise break-up of cumulative FDI inflow. The Singapore FDI inflow was given in Table 3 shows an annual exponential compound growth rate of 22.7 percent from 157,758.64 million in December 2008 to 1,086,273 million in December 2018, its gross cumulative inflows rose. The flow of FDI from Singapore moved at a slow rate until 2012, accounting for a small proportion of total inflows. However, there has been a spurt in the flow of FDI from this country since signing the comprehensive economic agreement with Singapore. In the following years, the FDI flow from Singapore registered enormous growth, with a slight exception in those years. In recent years, Singapore has taken the top spot for FDI flow by relegating Mauritius to the second position, total FDI inflows in 2018 have increased to 37.37 percent.

To sum up, Singapore has become India's most significant investor in FDI flows. At a 1 percent level of significance, the value of R-square is 78.5 significant. Because of the time factor, which is an independent variable, it shows a 78.5 percent variation. The F value is also significant at the 1 percent significance level. During the study era, it suggests a large fluctuation in its flow.

Fdi From Japan: Japanese FDI has been predominantly in the aerospace, electrical equipment, telecommunications, chemical, and pharmaceutical industries in India. The presence of Japanese firms in India has been gradually growing. There were 1,229 Japanese companies registered in India as of October 2015, an increase of 6 percent compared to 2014. As of February 2016, there were also a total of 4,417 Japanese business establishments operating in India, an increase of 14 percent compared to the previous year.6In the next five years, the governments of India and Japan signed an agreement to double Japanese investment in Indian companies and improve two-way trade. The plan was categorised into five broad areas: the creation of selected townships in India, promotion of investment and development of infrastructure, further development and cooperation in IT, enhancement of cooperation in strategic sectors, and economic integration in the Asia-Pacific region.

Table 6. Trend Analysis of Foreign Direct Investment from Japan from January 2008 to December 2018

Year	FDI from Japan	Total FDI	%Growth Per Preceding Year	% Share of out of Total
2008	16,976.32	1595295.00	-38.83	1.06
2009	60,943.17	1309799.00	258.99	4.65
2010	58,578.58	960150.00	-3.88	6.10
2011	143,486.13	1599349.00	144.95	8.97
2012	103,644.23	1215914.00	-27.77	8.52
2013	82,344.24	1294825.00	-20.55	6.36
2014	142,683.79	1753134.00	73.28	8.14
2015	110,843.79	2525614.00	-22.32	4.39
2016	388,098.00	311,643,9.7	250.13	12.45
2017	113,515.50	282,767,9.5	-70.75	4.01
2018	174,715.90	290,695,2.3	53.91	6.01
Total	1,395,829.65	21105152		
ECGR	20			
Constant value		31225.58		
Reg. coefficient		.200		
R-square		.569		
F-value		10.59		
P-value		.011		

Source: various newsletters from 2008 to December 2018

The country-wise FDI inflow analysis shows that Japan is India's fourth-largest investor. The share of Japanese

FDI inflows ranged from 4.01% to 12.45% of India's total FDI inflows. Nevertheless, its share of total FDI in India has shown a growing trend since 2009. The R2 value indicates that due to changes in time factor and F value, is 56.91, which is significant at 1 percent significance level.

Fdi From Netherland: In July 1988, the Indian government signed with the Netherlands the Avoidance of Double Taxation Prevention Fiscal Evasion Deal. Furthermore, as of May 2012, the Convention between India and the Netherlands on the Prevention of Double Taxation and the Prevention of Fiscal Evasion was amended.7 The Bilateral Investment Promotion and Security Agreement between the Republic of India and the Kingdom of the Netherlands for the Promotion and Protection of Investments was signed on 6 November 1995 and entered into force on 1 December 1995.

Table 7. Trend Analysis of Foreign Direct Investment from the Netherlands from January 2008 to December 2018

Year	FDI from Nether-Land	Total FDI Inflows	%Growth over the Preceding Year	% Share out of Total
2008	42,813.50	1595295.00	53.48	2.68
2009	40,056.60	1309799.00	-6.44	3.06
2010	52,061.05	960150.00	29.97	5.42
2011	58,889.06	1599349.00	13.12	3.68
2012	89,526.74	1215914.00	52.03	7.36
2013	125,318.06	1294825.00	39.98	9.68
2014	198,192.65	1753134.00	58.15	11.31
2015	192,548.62	2525614.00	-2.85	7.62
2016	200,993.80	3116439.70	4.39	6.45
2017	212,468.51	2827679.50	5.71	7.51
2018	232,330.90	2906952.30	9.35	7.99
Total	2,425,737.18	21105152		
ECGR	21.9			
Constant value		29732.41		
Reg. coefficient		0.219		
R-square		.929		
F-value		105.30		
P-value		.000		

Source: Various newsletters from 2008 to December 2018

During the study period, its share of total FDI flow ranged from 11.31 percent to 2.68 percent. The research observed the highest proportion of flows in 2014. But it declined after 2010 and began to grow again from 2012 to 2014. Its share of total FDI initially showed a declining trend until 2008. However, its share of total FDI has increased thereafter and it has risen from 2.68 percent in 2008 to 7.99 percent in 2018. The R square value indicates that a 92.9 percent shift in FDI inflow has been an account of being an independent time factor variable and the F value is significant at a 1 percent significance level.

Fdi From USA: The Agreement between the Government of the United States of America and the Government of the Republic of India on the prevention of double taxation and the prevention of tax evasion concerning income taxes, which entered into force on 18 December 1990, was concluded after the two Contracting States had told each other that the procedures laid down in their bribery laws had been completed. In 2008, the United States and India held two fruitful rounds of exploratory talks on a treaty that would include binding legal provisions on the treatment of one nation and the investment of another country. A press release by the Indian Ministry of Commerce and Industry dated September 21, 2011, indicated that the negotiation of a BIT with the US was almost complete.

Table 8. Trend Analysis of Foreign Direct Investment from the USA from January 2008 and December 2018

Year	FDI from USA	Total FDI Inflows	%Growth as Per Preceding Year	% Share out of Total
2008	75,419.79	1595295.00	107.29	4.73
2009	98,730.55	1309799.00	30.91	7.54
2010	64,824.03	960150.00	-34.34	6.75
2011	47,127.75	1599349.00	-27.30	2.95
2012	33,832.89	1215914.00	-28.21	2.78
2013	44,781.75	1294825.00	32.36	3.46
2014	101,476.12	1753134.00	126.60	5.79
2015	252,420.00	2525614.00	148.75	9.99
2016	176,027.50	3116439.00	-30.26	5.65
2017	141,886.10	2827679.50	-19.40	5.02
2018	189,911.00	2906952.30	33.85	6.53
Total	1,036,526.48	21105152		
ECGR	11.5			
Constant value		45460.21		
Reg. coefficient		0.115		
R-square		0.2977		
F-value		3.38		
P-value		0.103		

Source: Various Newsletters from 2008 to December 2018

The flow of FDI from the USA was given in Table 6 from 2008 to 2018. FDI from the USA has fluctuated widely throughout this period. The flow of FDI from the USA has increased at an annual exponential growth rate of 14.79 percent, despite significant variations on a year-by-year basis. Recently, there was a substantial increase in US FDI, with '101476.12 million in 2014 and' 252420 million in 2015, showing a rise of 126.60 percent and 148.75 percent respectively over the previous year. In

2018, it rose by 33.85 percent. Its share of the total flow of FDI ranged from 9.99 percent to 2.78 percent during this period. During this time, its share of total FDI also fluctuated dramatically from year to year. At an exponential 11.5 percent growth rate, the Indian economy is receiving US FDI. The R2 value shows that the disparity in FDI inflow from the USA of 29.77% was due to the time factor change with an F value of 3.38.

CONCLUSION

An analysis of the origin of FDI inflows into India shows that the sources of FDI inflows into India have been broadened by the new policy initiatives introduced. Over 180 countries contributed to FDI inflows in 2019, compared to just 15 in 1991. The percentage shares of FDI inflows from the top five countries underwent a compositional shift in favour of Singapore, Mauritius, and the United States over the period 2008 to 2018, comprising 30.49%, 22.79% and 7.98% of total FDI inflows, respectively. These countries contribute about 60% of India's FDI inflows. In 1990, only six countries, including the United States, the United Kingdom, Germany, Japan, France and the Netherlands, accounted for more than two-thirds of India's overall FDI inflows. The US has played an important role in FDI inflows to India since 1991, accounting for 19.31 percent of total FDI inflows. With the liberalisation of the Indian economy, except the United States, the United Kingdom, Germany, Japan, Italy, and France, which were now not only the key investors, but also during the pre-liberalization period, Mauritius, South Korea, Malaysia, the Cayman Islands, and many other countries appear mainly on the list of major investors.

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Antidiabetic Activity of the Bark of Indian Cherry, *Cordia dichotoma*

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ABSTRACT

Cordia dichotoma Forst. has been used in the management of diabetes in traditional medicine. However, the antidiabetic activity of the methanolic extract of *C. dichotoma* (MECD) bark has not been reported so far. In this study, the antidiabetic activity of *C. dichotoma* was assessed in alloxan-induced diabetic Wistar rats. The acute toxicity study indicated that the MECD was safe up to the dose level of 2000 mg/kg body weight. In oral glucose tolerance (OGT) test, the pre-treatment of MECD showed partial protection from hyperglycemia induced by a glucose load (2 g/kg, body weight) in rats at the dose levels of 250 and 500 mg/kg, body weight. The MECD significantly reduced the blood glucose levels in the alloxan-induced diabetic rats at the dose levels of 250 & 500 mg/kg, body weight as compared to normal control animals. Analysis of biochemical parameters and histopathological investigations also demonstrated the antidiabetic potential of the MECD with significant improvement of biochemical parameters including body weight, serum lipid profile and antioxidant enzymes/ biomarkers in comparison to the normal control. The activities of the MECD (500 mg/kg body weight) were comparable to some extent with that of the standard drug, glibenclamide (5 mg/kg). Our study scientifically validates the folkloric claim as well as traditional uses of *C. dichotoma* as antidiabetic medicine. It is suggested that the antidiabetic activity of *C. dichotoma* may be due to the presence of phenolic phytoconstituents or plant flavonoids in the methanolic bark extract.

KEY WORDS: CORDIA DICHOTOMA, METHANOLIC EXTRACT, ANTIDIABETIC ACTIVITY, FLAVONOIDS, ANTIOXIDANT PROPERTY.

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INTRODUCTION

Diabetes mellitus (or diabetes) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemic condition of diabetes may lead to several health complications including cardiovascular (cardiomyopathy), neurological (neuropathy), renal (nephropathy) and ocular (retinopathy) (Junejo et al., 2020a; Junejo et al., 2020b). In diabetic patients, the hyperglycemic condition is mainly because of the decreased insulin secretion due to the abnormal

functioning of insulin producing beta cells (present on the islets of Langerhans in pancreas) which thereby fail to produce enough insulin resulting in unstable blood glucose level followed by the occurrence of insulin resistance by metabolizing tissues (Tanwar et al., 2020; Yeh et al., 2003). The prevalence of diabetes is increasing with the global rise of obesity and lifestyle disorders.

According to World Health Organization (WHO) reports, it has been estimated that there were 422 million adults living with diabetes mellitus with 1.6 million deaths each year globally. The type 2 diabetes has been accounted for the majority (> 90-95%) of diabetes with approximately 1.5 million death annually (WHO, 2020). Various synthetic hypoglycemic agents have been used to control the elevated blood sugar level in patients with diabetes mellitus. Some common hypoglycemic agents approved by FDA include sulfonylureas, biguanides, thiazolidinediones and so on. Despite the availability of many effective oral hypoglycemic agents, diabetes is still life-threatening because of the limited clinical efficacy of existing drugs. Some common side effects that are associated with synthetic antidiabetic drugs include weight gain, weakness, fatigue, headache, palpitation, increased LDL chlosterol level etc. (Tanwar et al., 2020).

Plant-based traditional remedies have been used for the treatment of human diseases for thousands of years. About 80% of world population rely on traditional herbal medicines for primary health care. Traditional medicines derived from plants play a significant role in the management of a variety of human disorders including cancer, neurological disorders, diabetes and pain and inflammatory disorders, just to name a few. WHO has recommended the antidiabetic evaluation of traditional plant-based remedies or herbal preparations because they are effective with less or no toxicities as compared to synthetic antidiabetic drugs. Moreover, herbal medicines have the property of synergistic action due to the presence of a variety of active constituents in a single drug/ medicinal preparation (Jaiswal, 2016). Many indigenous Indian medicinal plants have been found to be useful in the treatment of diabetes mellitus. Ayurveda, Unani and Siddha, are the notable systems of medication documented in ancient practice basically utilizing plants/ plant-based preparations as medicines for curing human ailments/ diseases like diabetes. In view of their traditional and ethnopharmacological importance, herbal medicine may have potential role in the management of diabetes as well as its complications.

Cordia dichotoma Forst. (also known as Indian cherry) belonging to the family Boraginaceae is an average-sized tree of tropical and subtropical origin. It is widely found in Sri Lanka, India, and other tropical regions of the world. The use of this plant has been on ancient practice for the management of a variety of human disorders. It is also an important plant species found in the traditional Indian system of medicine including Ayurveda, Unani and Siddha. Seeds of *C. dichotoma* are used for the treatment of various inflammatory disorders. Fruits are used as expectorant, astringent, laxative and anthelmintic.

Some common ethnomedinal uses of *C. dichotoma* includes antidiabetic, immunomodulator, diuretic, anthelmintic, wound healing, antiulcer, gastro-protective, anti-inflammatory, antileprotic, antidiabetic, and hepatoprotective and antioxidant activities. The bark of *C. dichotoma* has been reported to possess betulin, α-amyrins, octacosanol, β-sitosterol, lupeol-3-rhamnoside, β-sitosterol-3-glucoside, hentricontanol, taxifolin-3,5-dirhmnoside, α-amyrin, hentricontane and hesperitin-7-rhamnoside (Hussain et al., 2020). There has been no scientific study on the the antidiabetic activity of the *C. dichotoma* bark previously reported in literature. The present study was, therefore, aimed at investigating the antidiabetic activity of the methanolic extract of *C. dichotoma* bark with a view to justify the traditional use of the plant in the treatment of diabetes.

MATERIAL AND METHODS

Drugs and chemicals: Alloxan monohydrate was purchased from Sigma-Aldrich, Mumbai, India. Glibenclamide was procured as a gift sample from Sun Pharmaceutical Industries Ltd., India. All other chemicals, reagents and solvents used were of analytical grade.

Plant material, extraction and phytochemical screening: The barks of *C. dichotoma* Forst. were collected during the month of April-May, 2012 from the Duhai forest of Ghaziabad, Uttar Pradesh, India. The plant material was identified from CSIR-National Institute of Science Communication and Information Resources (CSIR-NISCAIR), New Delhi. A voucher specimen (NISCAIR/ RHMD/ Consult/2012-13/2025/33) of the bark of *C. dichotoma* was submitted at the herbarium for future reference. The shade-dried barks of *C. dichotoma* were pulverized to coarse powder, defatted using petroleum ether, and extracted with methanol. The methanolic extract was subsequently evaporated to dryness and the concentrated extract so obtained was preserved in a refrigerator at 4 oC for further use. The percentage yield of the methanolic bark extract of *C. dichotoma* (MECD) was found to be 7.11% w/w on dry weight basis. The MECD was subjected to preliminary phytochemical screening to identify the presence of phytoconstituents as per the standard procedure previously described in literature (Ajiboye et al., 2020; Junejo et al., 2018).

Experimental animals: Adult Wistar albino rats of either sex weighing around 300-330 g were obtained from the Institutional Animal House for the experimental study. Animals were acclimatized to the standard laboratory conditions (temperature: 25 ± 2 °C, relative humidity: 50 ± 5 %) with a 12 h light/12 h dark cycle for a week before the beginning of experiments, and were provided with free access to the standard pellet diet and drinking water ad libitum. The experimental protocol was approved by the IAEC vide approval no. IAEC/DU/58 dated. 24/09/2013.

Acute oral toxicity:The acute toxicity test of MECD was performed on Swiss albino mice (40-45 g) as per the OECD guidelines (Junejo et al., 2014b). After overnight fasting, the animals were given a fixed maximum dose 2000 mg/kg body weight via intraperitoneal route of administration. Animals were under observation for 48 hours for signs of mortality or morbidity or death. One group was maintained as normal control and was given only vehicle.

Oral glucose tolerance (OGT) test: Overnight fasted rats were randomly divided into five groups with six animals each. Group 1 rats were given normal saline by oral route. Group 2 animals were administered glucose (2 gm/kg body weight) load orally. Group 3 and group 4 rats received MECD (250 and 500 mg/kg body weight, respectively) by oral route followed by the administration of glucose (2 gm/kg body weight) load after 30 minutes. Similarly, group 5 rats received glibenclamide (5 mg/kg body weight) followed by the glucose (2 gm/kg body weight) load. Blood was collected from the tail vein of animals at 30, 60, 90 minutes interval after the glucose administration. The blood glucose level was measured with the help of a glucometer (Lifescan, Milpitas, CA) (Amira et al., 206; Junejo et al., 2020b).

Evaluation of hypoglycemic activity: Diabetes was induced in overnight fasted rats withalloxan monohydrate (120 mg/kg body weight, in normal saline) administered by a single intraperitoneal injection. The animals confirmed as diabetic (after 72 h of alloxan injection) by the elevated plasma glucose levels (> 150 mg/dl) was used for the experiment. The animals were randomly divided into five groups of six rats each. Group 1 animals received normal saline orally. Group 2 received alloxan monohydrate (120 mg/kg body weight) by intraperitoneal route. Group 3 and group 4 received alloxan monohydrate (120 mg/kg body weight) intraperitoneally followed by the oral administration of MECD (250 and 500 mg/kg body weight, respectively). Similarly, group 5 received alloxan monohydrate (120 mg/kg body weight) followed by the standard drug, glibenclamide (5 mg/kg body weight) (Mishra and Garg, 2011; Raut and Naresh, 2006).

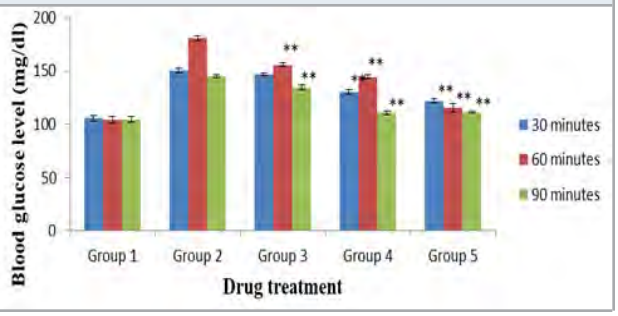
The blood samples were collected from the tail-tip of animals for measuring the blood glucose levels on 1st, 7th, 14th and 21st day. The blood glucose level was determined using the glucometer and the results were expressed as mg/dl (Banerjee et al., 2017; Junejo et al., 2020a).

Initial and final body weights were measured on 0 and 21st day (Junejo et al., 2017). The serum was separated by centrifugation (2000 rpm, 10 minutes) for the estimation of various biochemical parameters. Serum lipid profile was estimated using commercially available kits. Triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL) were determined (Junejo et al., 2014a). The levels of different antioxidant enzymes and/or markers were also determined. The activities/ levels of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and malonaldehyde (MDA) were measured. Malonaldehyde is formed in vivo as a result of lipid peroxidation in the tissue under oxidative stress (Junejo et al., 2017; Chaulya et al., 2010).

Histopathological investigation: At the end of 21 days of treatment, the animals were fasted for 12 hours, anaesthetized and sacrificed. Pancreas of rats from control, diabetic control, MECD treated, and standard drug treated (glibenclamide) groups were quickly removed and processed for histopathological studies. Pancreatic tissues removed from control and treated rats were washed in saline, the sections stained in haematoxylin-eosin were observed under a light microscope for histopathological investigations. Tissues were further fixed in Hollande-Bouin fixative for 48 hours. Fixed tissues were processed for paraffin embedding (Junejo et al., 2020 c).

Statistical analysis: Results are presented as mean ± standard error of mean (SEM). The one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to analyse and compare data. Statistical analysis was performed using the IBM SPSS 19.0 statistical software package, for Windows. Statistical differences at 1% ($p < 0.01$) level of probability between the groups were considered statistically significant.

Figure 1: OGT test. Values were expressed as mean ± SEM (n = 6). Activities of MECD and standard drug, glibenclamide are statistically significant at **p < 0.01 as compared to normal control. Group 1: Normal control; Group 2: Diabetic (alloxan) control; Group 3: MECD, 250 mg/kg; Group 4: MECD, 500 mg/kg; Group 5: Glibenclamide, 5 mg/kg.

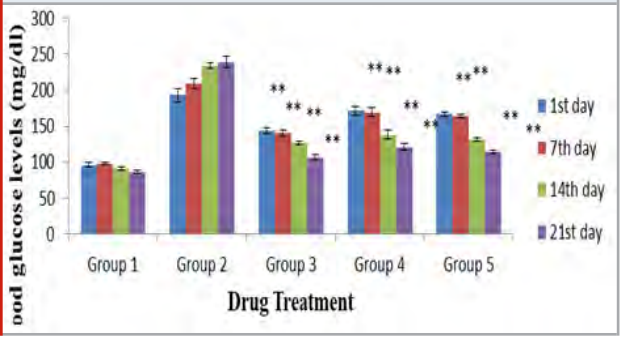


RESULTS AND DISCUSSION

Preliminary qualitative phytochemical analysis showed the presence of alkaloids, steroids, phenolic compounds, flavonoids, tannins, saponins and carbohydrate in the MECD.The animals showed no signs of adverse effects during the period of observation. No gross behavioural changes like drowsiness, restlessness, writhing, convulsions and symptoms of toxicity and mortality were observed in animals up to 48 hours of experimentation. The body weight and food consumption were normal as compared to control animals. The MECD was found to be safe up to the test dose of 2000 mg/kg body weight. Figure 1 displays the results of OGT test. The MECD treated groups showed significant ($p < 0.01$) reduction of blood glucose levels at both the doses (250 and 500 mg/

kg) as compared to the normal control group. Similarly, the standard, glibenclamide (5 mg/kg) treated group also exhibited significant ($p < 0.05$) activity in comparison to normal control.

Figure 2: Effect of MECD on blood glucose level in alloxan-induced diabetic rats. Values indicate mean ± SEM (n = 6). Activities of MECD and standard drug, glibenclamide are statistically significant at **$p < 0.01$ as compared to normal control. Group 1: Normal control; Group 2: Diabetic (alloxan) control; Group 3: MECD, 250 mg/kg; Group 4: MECD, 500 mg/kg; Group 5: Glibenclamide, 5 mg/kg.



Results of the effect of MECD on blood glucose level of alloxan-induced diabetic rats are depicted in Figure 2. After treatment with MECD, the blood glucose levels were significantly ($p < 0.01$) reduced at both the doses (250 and 500 mg/kg) as compared to the diabetic control group. The glibenclamide (5 mg/kg) treated group also showed significant ($p < 0.01$) reduction in the blood glucose level when compared to normal control group.

After 21 days of treatment with MECD (250 and 500 mg/kg), the body weight of animals were significantly ($p < 0.05$) increased as compared to diabetic rats. The effects of MECD on body weight and lipid profile of diabetic rats are presented in Table 1. In diabetic rats, the levels of TG, TC, and LDL were significantly increased, and the HDL level was significantly decreased. In MECD (250 and 500 mg/kg body weight) treated groups, the TG, TC and LDL levels were significantly ($p < 0.01$) reduced and the HDL level was significantly ($p < 0.01$) increased as compared to diabetic control rats. The activities of MECD at the dose of 500 mg/kg body weight were somewhat comparable with that of glibenclamide (5 mg/kg).

Table 1. Effect of MECD on body weight and lipid profile of diabetic rats

Group	Body weight (g)		Lipid profile (mg/dl)			
	0 day	21st day	TG	TC	HDL	LDL
Group 1 (Normal control)	300.23 ± 3.56	302.38 ± 2.49	85.32 ± 2.45	156.71 ± 4.10	36.14 ± 3.45	98.14 ± 4.41
Group 2 (Diabetic control)	300.12 ± 3.86	270.90 ± 3.46	215.14 ± 4.61	250.34 ± 5.20	36.10 ± 4.20	189.51 ± 4.20
Group 3 (MECD, 250 mg/kg)	298.34 ± 2.43**	290.30 ± 4.21**	147.21 ± 2.35**	160.44 ± 4.73**	43.71 ± 5.62**	118.21 ± 7.21**
Group 4 (MECD, 500 mg/kg)	300.23 ± 5.42**	292.96 ± 4.72**	38.34 ± 3.91**	148.31 ± 4.00**	49.32 ± 4.19**	102.42± 6.34**
Group 5 (Glibenclamide, 5 mg/kg)	299.24 ± 3.79**	298.59 ± 3.72**	116.56 ± 7.16**	147.32 ± 2.16**	56.11 ± 4.10**	76.34 ± 3.62**

Results are expressed as mean ± SEM (n = 6); **$p < 0.01$, compared to normal control. TG: Triglyceride, TC: Total cholesterol, HDL: High density lipoprotein, LDL: Low density lipoprotein. The MECD restored significantly ($p < 0.01$) the levels/ activities of GSH, SOD and CAT at both the doses (250 & 500 mg/kg body weight) as compared to the normal control. Additionally, the MECD significantly ($p < 0.01$) improved the levels of LPO marker component i.e., MDA in comparison to the normal control group. Results of the effects of MECD on antioxidant enzymes/ markers of diabetic rats are displayed in Table 2. Results also reveal that the antioxidant activity of MECD (500 mg/kg body weight) was comparable with that of the standard drug, glibenclamide (5 mg/kg body weight).

Histopathological investigations of pancreas of alloxan-induced diabetic rats (Figure 3) exhibited pathophysiological features with the reduction in the number of islets, damaged β-cell population and extensive necrotic changes followed by fibrosis and atrophy (Slide 2). In the normal control group, normal cellular population in the islets of Langerhans were observed (Slide 1). MECD (250 and 500 mg/kg) (Slide 3 and Slide 4, respectively) and glibenclamide (5 mg/kg) (Slide 5) treated rats restored the necrotic and fibrotic changes and also increased the number and the size of the islets.

Cellular oxidative stress (OS) induced by the reactive oxygen species (ROS) produced from the action of

free radicals in the biological matrix may be increased abnormally during diabetes, causing an imbalance between the cellular metabolism and the antioxidant system of the body. The oxidative stress produces inflammatory cascades that damage the cellular components such as beta cells of islets of Langerhans (Tanwar et al., 2020).

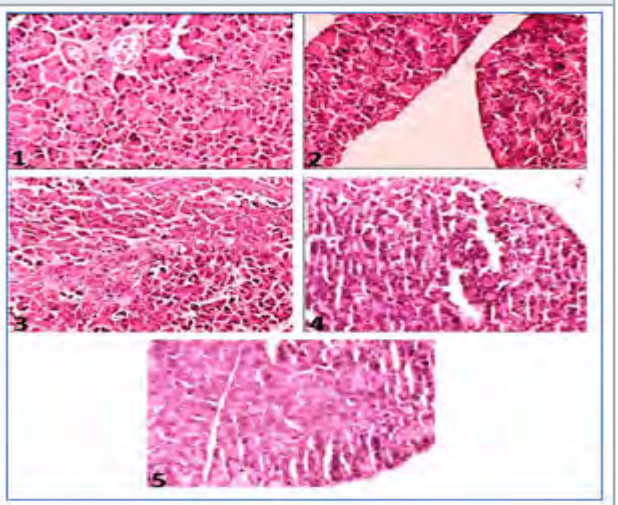
Further oxidative stress is undoubtedly claimed to have significant involvement in the pathogenesis of chronic diabetic mellitus along with various disease complications. The oxidative stress can be reduced to a considerable extent by the action of various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH) and glutathione peroxidase (GPx) (Junejo et al., 2017, Debasis et al., 2010). Besides hypoglycemic activity, the antioxidant activity of herbal drugs can help reduce the long term complications of diabetes. Restoring the levels of antioxidant enzymes, herbal medications could act as free-radical scavengers and eventually prevent generation of ROS and OS induced cellular damages. Many studies have investigated the antioxidant potential of plant polyphenols and flavonoids.

Table 2. Effect of MECD on antioxidant enzymes/ markers of diabetic rats

Groups	GSH (µmoles of GSH/ mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nmoles of MDA/ mg of protein)
Group 1 (Normal control)	4.99 ± 0.17	3.79 ± 0.13	5.09 ± 0.18	3.53 ± 0.17
Group 2 (Diabetic control)	3.11 ± 0.14	2.20 ± 0.26	2.01 ± 0.25	5.29 ± 0.26
Group 3 (MECD, 250 mg/kg)	4.82 ± 0.18**	4.08 ± 0.25**	4.85 ± 0.23**	4.83 ± 0.15$
Group 4 (MECD, 500 mg/kg)	5.58 ± 0.12**	4.75 ± 0.20**	5.07 ± 0.13**	4.16 ± 0.19**
Group 5 (Glibenclamide, 5 mg/kg)	4.25 ± 0.18**	3.93 ± 0.25**	3.62 ± 0.24**	4.13 ± 0.18**

Results are expressed as mean ± SEM (n = 6); **p < 0.01, compared to normal control. GSH: Reduced glutathione, SOD: Superoxide dismutase, CAT: catalase, MDA: Malonaldehyde.

Figure 3. Photographs showing histopathology of pancreas of experimental rats. Slide 1: Treated with normal saline, Slide 2: Treated with alloxan (120 mg/kg), Slide 3: Treated with MECD (250 mg/kg), Slide 4: Treated with MECD (500 mg/kg), Slide 4: Treated with standard drug, glibenclamide (5 mg/kg).



Phenolic phytoconstituents and flavonoids have been attributed to exhibit pharmacological effects against heart diseases, cancer, neurological disorders, diabetes, inflammatory disorders and so on owing to their radical scavenging actions (Sekhin-Loodu and, Rupasinghe, 2019; Shaheen et al., 2017). In this study, the antioxidant potential of MECD may be the underlying reason behind its hypoglycaemic action. Phenolic compounds and flavonoids of MECD could prevent cellular oxidative stress and eventual tissue damages associated with diabetes. However, the probable mechanism of hypoglycemic action may be due to the potentiating effect of insulin either by stimulating the pancreatic insulin secretion from the β-cells of the islets of Langerhans or by its action on the body tissues.

CONCLUSION

It is concluded that the metabolic extract of *C. dichotoma* (MECD) bark possesses antidiabetic activity in alloxan-induced diabetic rats. Our study scientifically validates the folkloric claim as well as traditional uses of *C. dichotoma* as antidiabetic medicine. It could be attributed that the antidiabetic activity of *C. dichotoma* may be due to the presence of phenolic phytoconstituents or

plant flavonoids in the methanolic bark extract. Further studies can be carried out in order to explore the specific phytochemical(s) responsible for the antidiabetic potential *C. dichotoma*.

Conflict of Interest: None

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Occupational Stress in Relation to Teacher Self - Efficacy and Spiritual Intelligence of Women Teachers

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ABSTRACT

Education is a powerful instrument and catalyst of human development and empowerment for achievement of a better and high quality of life. The role of teacher is very important in imparting the knowledge and implementing the plans and programmes of the nation. But in present era of growing complexities the teacher is one of the most exposed person to stress and strain. It is an established fact that performance of a teacher mainly depend upon his/her psychological state of mind. The stress results into teacher's physical, mental illness. Particularly, in case of women teachers, while performing dual role that is balancing work and family women they face various psychological and social problems. So, women teachers should be mentally strong enough to deal with all the challenges related to professional and personal life. The present study was conducted with an objective to find out the relationship between occupational stress, teacher self-efficacy and spiritual intelligence of women teachers. The sample of the study was 500 women teachers randomly selected from the five districts from the state of Punjab. The findings of the study reveal that- A significant and negative relationship was found between occupational stress, teacher self-efficacy and spiritual intelligence of women teachers. It is quite apparent from the regression model summary that teacher self-efficacy and spiritual intelligence of women teachers both independently as well as conjointly predicts occupational stress. Hence, it is obvious from the results that the teacher self-efficacy and spiritual intelligence are the most significant and influential contributor in predicting occupational stress of women teachers.

KEY WORDS: OCCUPATIONAL STRESS, TEACHER SELF-EFFICACY, SPIRITUAL INTELLIGENCE.

INTRODUCTION

The whole advancement of our civilizations is in fact, based on the advancement of our education. The aims, processes and valuation of education are primarily associated with teaching. Sharma (2018) points out that national development and a society prosperous with knowledge all begins from its teachers. Teaching is one of the most significant profession of world. For the qualitative improvement of education, there is a need of effective teachers as the edifice of education depends on effective teaching. The most important factor of education is teacher, his personal qualities, his educational qualifications, his professional training and the place that he occupies in school as well as in the community. Hence it is important for the teacher's life to be satisfied. But in present era of growing complexities the teacher is

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one of the most exposed person to stress and strain. It has been found that teaching is one of the jobs which is full of stress (Borg & Riding, 1991; Brouwers & Tomic, 1999; Sharma & Marwaha, 2020).

It is an established fact that performance of a teacher mainly depend upon his/her psychological state of mind. Surekha (2016) Studied work life balance of 90 married women working in software Industries in Bangluru (India) and found that employment of women affects health both physiologically and psychologically. But while performing dual role that is balancing work and family women teacher face various psychological and social problems. Most women do not have responsibility only in one domain anymore; they have to balance the competing demands of both work and family domain (Bicaksiz 2009). Working women's problems are aggravated by their multiple role expectations which we find in Indian society. Today, women have to perform multiple roles as of wife, mother, homemaker, worker and a citizen. A working women taking up a job outside home also has to look after domestic work along with her official work. They dual responsibility has over burdened working women there by leading to multidimensional problems (Pandya and Thakkar, 2009).

Too much stress on mind and body can make working women feel miserable worried, sad and ill. Particularly, teacher stress has increasingly been recognized as a widespread problem in different educational settings. (Boyle, Brog, Falzon and Baglioni 1995; Dick and Wagner, 2001; Kyriacou, 2001) compared to the general population, teacher are at risk for higher levels of psychological distress and lower level of job satisfaction (Travers and copper, 1996; Schonfield, 1990). Brog (1990) also reported that up to one third of teachers perceive their occupation as highly stressful. Many job conditions caused stress among working women. These job conditions include little control over work; role ambiguity and conflict; poor relationships with co-workers and supervisors; heavy workload demands; job insecurity and work that are narrow, repetitive and monotonous. Besides this, delayed salaries, under paid, extra duties like election duties, pulse polio, social survey etc. produce stressful situations for women teachers. This stress results into teacher's physical, mental illness (Sharma & Kaur, 2017; Sharma & Marwaha, 2020).

Sharma and Kaur (2017) conducted a study on a sample of 500 married women teachers teaching in schools and colleges in the state of Punjab, India. Data was collected with the help of Psycho-Social Problems of Educated Working Women Scale by Hundal (2002). The major findings of the study reveal that school and college women teachers do not differ significantly on psycho-social problems. Women teachers with age (<35 years and >35 years) do not differ significantly on psycho-social problems. School and college women teachers do not differ significantly on psycho-social problems due to interactional influence of category of teachers (school & college) and age (<35 years & >35 years). These psychological and social problems affect the physical and psychological well being of teachers it influences teaching effectiveness and performance of a teacher.

So, in order to teach effectively, teacher must not only feel psychologically and emotionally comfortable, but they must also have some sense of belief that they can make a difference to the lives of children they are teaching. For nurturing self-efficacy in students' qualitative education needs effective teachers. Effective teachers are those who are committed, enthusiastic, intellectual and emotionally stable and with a high level self-efficacy. Self-efficacy has its root in social cognitive theory of Bandura, particularly in the context of cognitive behaviour modification (Sharma & Kaur, 2017; Sharma, 2020).

Tecaher self-Efficacy is an important application of Bandura's (1977) social cognitive theory to educational setting (Sharma, 2018). Teachers having efficacious outlook fosters their intrinsic interests and deep engrossment in teaching activities such teachers holding high confidence in their capabilities approach difficult task as challenges to be mastered rather than to be avoided as threats. They set to themselves challenging goals and maintain strong commitment to the fact the higher sense of self-efficacy in human beings includes great effort Persistence and resilience. So, modern system needs teachers having strong self-efficacy and health mental health. A teacher with high efficacy exhibits less anger , less stress, use of fewer control tactics, use of cooperative learning and more enthusiastic towards teaching (Sharma & Kaur, 2015; Sharma,2020). Teacher with high self-efficacy teach well due to their self-confidence and quality of motivating students (Khurshid, Quasism and Ashraf (2012).

Teachers with high self-efficacy are more motivated than the teachers with low self-efficacy. This motivation enhances their work. Such teachers provide an opportunity for student having low self-efficacy to learn a lesson from them. Akram and Khuwaja (2014) compared 100 working and 98 non working women of Pakistan and found that non -working women have higher level of depression and compared to working women. But married women working as manager, lawyers have more stress as compared to teachers, lecturers and doctors. A study was carried out with an objective to find out the contribution of emotional intelligence, occupational stress and self efficacy to job satisfaction.

Study was conducted on a sample of 398 secondary school teachers of Ondo state (Nigeria). Emotional intelligence questionnaire by Schutte et. al (1998); General Self -Efficacy scale' by Sherer et., al., (1982) and 'Job Satisfaction Scale' by steers (1991) and 'Occupational Stress Scale' by Hassan and Hassan (1998) were used as data collection instruments. It was found that emotional intelligence is more important than self -efficacy jointly predict job satisfaction but the contribution is emotional intelligence is more important than self -efficacy and

occupational stress do not predict job satisfaction among teachers (Akomolafe and Ogunmakin, 2014).

Another study conducted by Mohammadi and Mohammadipour (2015) the results indicated a significant relationship was found between self -efficacy, job performance and mental health. 'Job Performance Questionnaire' by Paterson (1991) 'NEO- Personality Inventory – 'Revised' by Kasta and Mc Cary (1992); General Health Questionnaire' by Goldenberg (1979) and 'Sherer Self-Efficacy Scale' by Sherer and Moddux (1982) were employed as tools for data collection 200 Iranian teachers were taken as a sample of the study. Whereas, Singh and Singh (2015) compared job satisfaction and self- efficacy of 82 regular and 118 contractual teachers of district Patna (India). Results reveal that teachers appointed on contract basis have poor job satisfaction and low self -efficacy than regular teachers. 'Job Satisfaction Scale' by Singh and Sharma (1990) and 'Self-Efficacy Scale by Singh and Narain (2014) were used for data collection. Qadini et al. (2015) explored the effect of teaching experience on occupational stress on a sample of 819 teacher of Mysore Karnatka (India) and found that teachers with an age group of 15-20 years of teaching experience have maximum stress as compared to >20 years of teaching experience. However teachers with <5 years, 6-10 years and 11-15 years of experience have less stress. 'The Occupational Stress Index' by Srivastava and Singh (1984) was used as data collection tool.

Rastegar and Moradi (2016) examined the relationship between Iranian English language teachers' job satisfaction, self - efficacy and their spiritual well being. 'Job Muhangi (2017) conducted study on 626 teachers of district, Mbarora, Uganda and found that male and mature teachers have strong sense of self- efficacy than female and young teachers. It was further found that self -efficacy and job satisfaction are correlates to turnover intentions. Sharma and Kaur (2017) found that school and college women teachers do not differ significantly on teacher self-efficacy. Women teachers generally have average level of teacher self-efficacy. Sharma (2018) found no significant difference in teacher self-efficacy of school and college women teachers with respect to locale. Sharma (2018) conducted a study on 250 married college women teachers and found a significant and negative relationship between teacher self-efficacy and psycho-social problems of college women teachers. It has been found that school women teachers with <35 years of age have significantly higher level of self-efficacy than college women teachers with <35 years of age. College women teachers with >35 years of age have significantly higher level of teacher self-efficacy than school women teachers with >35 years of age (Sharma, 2018).

Spiritual intelligence is concerned with the inner life of mind and spirit and it is related to being in the world. It is the ability of an individual to think about the world, one's self and spend one's life according to that (Ronel and Gan 2008). Spiritually intelligent people have the ability to experience heightened states of consciousness and can utilize spiritual resources to solve problems (Emmons, 2000). It is considered as a capacity for a deep understanding of existential questions and insights into multiple levels of consciousness. It has become an important part of our lives as well as workplace. Kaur, (2013) suggested that spirituality is considered as one of the key factors for the success of the educational organisations and ultimately for the professional life of the teachers. Kalantarkousheh et., al. (2014) also found that higher spiritual intelligence is associated with higher organizational commitment. A significant and positive relationship was found between spiritual intelligence and job performance (Utomo et al. 2014). When spiritual intelligence is high, a person appears to be intellectual and have proper behaviour (Zohar and Marshall, 2000).

Spiritual Intelligence is defined as the as ability to apply and embody spiritual aspects and qualities to promote daily functioning and wellbeing. Amram (2007) gave 7 major themes of Spiritual Intelligence like as Consciousness: (developed refined awareness and self-knowledge) Grace (Living in alignment with the sacred manifesting love for and trust in life) Meaning (Experiencing significance in daily activities through a sense of purpose and a call for service including in the face of pain and suffering) Transcendence: (Going beyond the separate egoist self into an interconnected wholeness) Truth (Living in open acceptance, curiosity, and love for all creation) Peaceful surrender to Self (True, God, Absolute, true nature) and Inner-Directedness(inner-freedom aligned in responsible wise action) (Wigglesworth, 2006).

Teacher as a human being and spiritual being possesses spiritual intelligence that helps him/her to deal effectively. Sreeja (2005) conducted a study on "Spirituality, emotional, maturity, and quality of life among university students" and found that there is significant difference between boys and girls in spirituality but no significant difference between boys and girls in emotional maturity and quality of life. It was further found that spirituality and emotional maturity are independent of religion, but significant correlation was found between spirituality and emotional maturity. Spiritual intelligence provides a sense of personal wholeness, goal and direction (Dincer, 2009).

Siswoyo et al. (2018) found that emotional intelligence and spiritual intelligence has positive and significant effect on organizational commitment. Another study conducted by Shahokhi et al. (2018) whose results shown that spiritual intelligence and education level can be considered as predictive variables for perceived stress ($P<0.001$). A significant relationship was between spiritual intelligence and perceived stress and the predictive role of spiritual intelligence was considered as spiritual intelligence is one of the effective strategies in reducing stress that can be promoted by nurses. Human capital is one of the important determinants of nation's growth and teachers are the key persons responsible for shaping the destiny of the nation. Teacher is the heart and core of an educational process. Teachers have a pivotal role in social reconstructions and in the transmission

of knowledge and experience of one generation to another. Thus, it is important that the teachers get a congenial environment at the work place as it is the teachers upon who rests the task of developing pupils. Effective teachers pave the way for an enlightened and productive society.

Self- efficiency refers to a belief in one's own capacity to organize and execute the course of action required to manage difficult and problematic situation. When an individual faces stressful situations, self - efficacy helps the individual to interpret such situations as challenges rather than threat. Thus teachers with high self-efficacy will be more successful in coping with stressful situations. There is scarcity of studies related to occupation stress in relation to teacher self-efficacy in the realm of women teacher especially in Punjab. Apart from teacher's self-efficacy, spiritual intelligence of a teacher is another important factor in teacher's ability to cope with stress. The qualities that are required in most of the context of teachers are that which makes them contended in challenging situations.ie. Spiritual intelligence. Researches by Litwinczuk and Groh (2007), Brillhart (2005), George (2006), Emmons (2000), Faribers, Fatemah and Hamidreza (2010),

Zhaleh and Ghonosooly (2017), Abbas, Bordbar, Moghadam ans Ali (2018) showed importance of spiritual intelligence in relation to life purpose, well being, personal security, happiness and ability to handle, adverse situations. The research gap identified in the study of related literature is that there are no studies that investigated into the relationship between occupational stress, teacher self-efficacy and spiritual intelligence of women teachers. Hence, the present study has been planned to study the relationship of occupational stress, teacher self-efficacy and spiritual intelligence especially in case of women teachers. It will also essential and beneficial for planners and educational authorities to consider the relationship between occupational stress, teacher self-efficacy and spiritual intelligence of school and college women teachers and should try to provide suitable environment in educational institutions so that the academic achievement of the students may be enhanced. Therefore, investigator made an attempt to study occupational stress in relation to teacher self-efficacy and spiritual intelligence of women teachers.

Statement of the Problem: Occupational Stress In Relation To Teacher Self-Efficacy And Spiritual Intelligence Of Women Teachers:Operational definitions of the terms used :Occupational stress- It is often stems from undue pressure and unexpected responsibilities that do not match with person's knowledge, skills or expectations that employee face during job (Jamal & Raheen, 2012).

Teacher self-efficacy: It refers to the belief in the efficiency of one's teaching, teaching methods and judgment of his capabilities to bring about desired changes in student learning (Sharma, 2017).Spiritual intelligence-Spiritual intelligence is the ability to behave with wisdom and intelligently, therefore maintaining internal and external peace, despite of the condition (King, 2008).

Objectives of the study: To find out significant relationship between occupational stress and teacher self-efficacy of women teachers . To find out significant relationship between occupational stress and spiritual intelligence of women teachers. To find out conjoint effect of teacher self-efficacy and spiritual intelligence on occupational stress of women teachers

MATERIAL AND METHODS

Research design, Participants of the study and Sampling technique- Research design may be defined as a plan or blue print describing the conditions and procedures for collecting and analyzing data (McMillan & Schumacher, 2010). The present study is a descriptive survey method. A sample of 500 women teachers teaching in government and self-financed schools and colleges from five districts of Punjab were selected randomly. Multistage randomization sampling technique was employed. Districts, the colleges, the schools, the teachers were selected by using random sampling technique.

Hypotheses of the study: There exists a significant relationship between occupational stress and teacher self - efficacy of women teachers. There exists a significant relationship between Occupational stress and spiritual intelligence of women teachers. The conjoint effect of teacher self-efficacy and spiritual intelligence on occupational stress of women teachers is higher than their individual effects.

Tools used: Teacher's Occupational Stress Scale by Jamal and Raheen (2012).Teacher Self-Efficacy Scale by Sharma (2017).Spiritual Intelligence Self Report Inventory (SISRI) by David B. King (2008)

RESULTS AND DISCUSSION

It can be seen from Table No. 1 that the coefficient of correlation between occupational stress and teacher self-efficacy of women teachers came as -0.35 which is significant at .01 level of confidence, meaning thereby that, a significant and negative relationship exists between occupational stress and teacher self-efficacy of women teachers. This implies that the women teachers who have higher level of teacher self-efficacy experience less occupational stress and the women teachers who have lower level of teacher self-efficacy experience more occupational stress. Thus, the above result leads to the acceptance of Hypothesis 1, "There exists a significant relationship between occupational stress and teacher self-efficacy of women teachers".

This implies that higher the level of teacher self-efficacy of women teachers; lesser will be the occupational stress. It may be due to the fact that strong sense of self-efficacy enhances the level of accomplishment and personal well being of person in a countless ways.

They approach difficult tasks as challenges rather than as threats (Sharma, 2018). This result of the study also corresponds with findings reported by Kumar (2013); Gupta and Kumar (2014); Zaki (2016); Sharma (2018) and Sharma (2020) as they found significant and negative relationship between teacher self-efficacy and occupational stress. The investigators could not find any study showing positive relationship between teacher self-efficacy and occupational stress. As, such a result is neither expected nor desired.

Table 1. Showing the coefficient of correlation between occupational stress and teacher self-efficacy of women teachers

Variables	category	N	r	Sig./NS
Occupational stress and teacher self - efficacy	women teachers	500	-0.35	Sig. at .01 level

To achieve above stated hypothesis, coefficient of correlation was calculated with product moment method between the scores of women teachers on the variables of occupational stress and spiritual intelligence and it came as -0.39 which is significant at .01 level of confidence which leads to the conclusion that a significant and negative relationship exists between occupational stress and spiritual intelligence of women teachers. This implies that the women who are spiritually more intelligent experience less occupational stress and the women teachers who are spiritually less intelligent experience more occupational stress.

Table 2. Showing the coefficient of correlation between occupational stress and spiritual intelligence of women teachers

Variables	category	N	r	Sig./NS
Occupational stress and spiritual intelligence	women teachers	500	-0.39	Sig. at .01 level

As, a significant and negative relationship was found between the scores of women teachers on the variables of occupational stress and spiritual intelligence, therefore Hypothesis 2 i.e., "There exists a significant relationship between Occupational stress and spiritual intelligence among women teachers" stands accepted. The findings of Zohar and Marshall (2001); Nelms (2005); Kumar and Pragadeeswaran (2011); Marzabadi, Hoshmandja and Poorkhalil (2013) ;Utomo et al. (2014); Zhaleh and Ghonsooly (2017); Abbas, Bordbar, Moghadam and Ali (2018);Shahokhi et al. (2018); Yahyavi, Narab and Yahyavi (2018) and Mathew, Shetty and Nair (2020) are in line with present findings as they researched that higher the level of spiritual intelligence lowers the stress level of a person.

Regression for predictive efficiency: 'The conjoint effect of teacher self-efficacy and spiritual intelligence on occupational stress of women teachers is higher than their individual effects'.

The effect of teacher self-efficacy on occupational stress of women teachers was found to be significant at .01 level (F(1, 498)= 70.98). The computed value of R2 of teacher self-efficacy and occupational stress of women teachers (YX1) is 0.125 which indicates that the contribution of teacher self-efficacy on occupational stress is 12.5%. The occupational stress of women teachers can be predicted with the equation. Occupational Stress = 144.97 -0.23 x teacher self-efficacyi.e. for every unit of increase in teacher self-efficacy, occupational stress of women teachers decrease .23.

The effect of spiritual intelligence on occupational stress of women teachers was found to be significant at .01 level (F(1, 498)= 87.43). The computed value of R2 of spiritual intelligence and occupational stress of women teachers (YX2) is 0.149 which indicates that the contribution of spiritual intelligence on occupational stress of women teachers is 14.9%. The occupational stress can be predicted with the equation: Occupational stress = 181.15-0.48 x spiritual intelligence i.e. for every unit of increase in spiritual intelligence, occupational stress of women teachers decrease .48. The conjoint effect of both teacher self-efficacy and spiritual intelligence on occupational stress of women teachers was found to be significant at 0.01 level of significance (F(2,497)=66.84). The computed value of R2 of occupational stress with teacher self-efficacy and spiritual intelligence (YX1X2) is 0.212 which indicates the contribution of teacher self-efficacy and spiritual intelligence on occupational stress of women teachers is 21.2%.

As %age variance (=21.2) of variables of teacher self-efficacy and spiritual intelligence conjointly on occupational stress of women teachers shows increase in its value from teacher self-efficacy (%age variance =12.5) and spiritual intelligence (%age variance =14.9), it indicates that the conjoint effect of teacher self-efficacy and spiritual intelligence on occupational stress of women teachers is higher than that of teacher self-efficacy and spiritual intelligence separately. The occupational stress of women teachers can be predicted with the equation : Occupational stress = 197.83-0.17 x teacher self-efficacy -0.39 x spiritual intelligence.

Hence, hypothesis 3 i.e., "The conjoint effect of teacher self-efficacy and spiritual intelligence on occupational stress of women teachers is higher than their individual effects" stands accepted.The investigator did not come across any study in favor of above said results. But the investigator could find studies such as, Anusiem, Okoiye and Emmanuel (2015) who found occupational stress has more predictive influence in teacher efficacy of secondary school teachers as compared to teaching anxiety. Similar

results were reported by Lu, Siu & Cooper, (2005); Cascio, Magnano, Elastico, Costantino, Zapparrata & Battiato, (2014) and Aggarwal, (2015) who found self-efficacy is a potent predictor of stress. Sharma (2020) also found that conjoint effect of teaching experience and psycho-social problems is higher as compared to their separate prediction in predicting the teacher self-efficacy of school women teachers.

Table 3

Variable	R	R^2	% Variance	F	Inference	Step-up Regression Equation
YX_1	0.353	0.125	12.5	70.98	Sig at 0.01 level	$Y=144.97-0.23X_1$
YX_2	0.386	0.149	14.9	87.43	Sig at 0.01 level	$Y=181.15-0.48X_2$
YX_1X_2	0.460	0.212	21.2	66.84	Sig at 0.01 level	$Y=197.83-0.17X1-0.39X_2$

Y - Occupational stress, X_1 - teacher self-efficacy, X_2 - spiritual intelligence

Conclusions of the study: A significant and negative relationship was found between occupational stress, teacher self-efficacy and spiritual intelligence of women teachers. It is quite apparent from the regression model summary that teacher self-efficacy and spiritual intelligence of women teachers both independently as well as conjointly predicts occupational stress. Hence, it is obvious from the results that the teacher self-efficacy and spiritual intelligence are the most significant and influential contributor in predicting occupational stress of women teachers.

Educational Implications of the study: Based on the findings of the study following educational implications has been drawn: Seminars and workshops should be organised to improve the level of self-efficacy and spiritual intelligence of women teachers. Workshops on yoga and meditation for women teachers should be organized to minimise the level of their occupational stress and increase the level of spiritual intelligence and teacher self-efficacy (Sharma, 2018).

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Seasonal Variation of Heavy Metals and Fish Diversity on Different Open Cast Coal Mine Pits of Satgram and Kajora Areas Raniganj, West Bengal, India

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ABSTRACT

Open cast pit (OCP) filled with surface runoff and groundwater recharge. A total of 40 PLs were enumerated and characterized to determine their nature, position, depth, area and comparative account in RCF during the period of 2014–2017. A 1-year study of physicochemical parameters of water and soil was recorded at 27 selected mine PLs to understand its quality. During the study period, the 14 most frequently cultured/naturally occurred fish species were collected and identified from the PL. PLs aged over 20–30 years turned naturally into wetland ecosystem harbouring a good amount of aquatic biota, excellent water quality and stabilized embankment. The present study is based on the relationship between seasonal variation of heavy metals and fish diversity. During the analysis it is found that there is a direct impact on heavy metal concentration in these OCPs during different seasons. It was observed that concentration level differed among OCPs due to their different geographical location. Monsoon is the most vulnerable season for heavy metals concentration and pit lakes of Satgram area were more contaminated than those of Kajora Area. As a result of it, fish diversity and fish production were high in Parasea OCP and Ghanshyam OCP than Damalia OCP and Ratibati OCP and it may also be stated that post monsoon is most productive season in terms of rich fish diversity and fish production. The main aim of the work was to find out the relationship between the seasons and heavy metals concentration and its impact on fish diversity. It also gave emphasis to find out the probable measures for improvement of fishery sector.

KEY WORDS: HEAVY METAL (HM); OPEN CAST PIT; PHYSIOCHEMICAL PARAMETER; SURFACE RUNOFF; FISH DIVERSITY; WETLAND.

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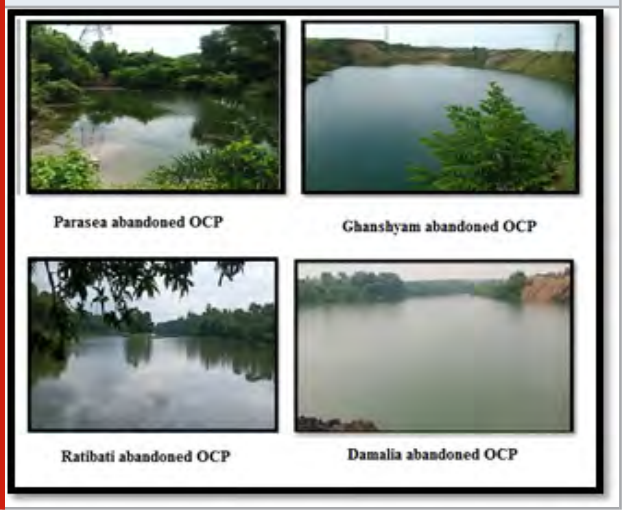
INTRODUCTION

In 1774 coalmining in India was first initiated in the Raniganj Coalfield, in Bengal province. Rich treasure of coal was found near Ethora, presently in Salanpur community development block by John Sumner and Suetonius Grant Heatly of the British East India Company. The exploration and mining operations were haphazard in early stage. Alexander & Co started regular mining

in 1820. In 1835, after the collieries had been bought by Prince Dwarkanath Tagore the field was led by Carr Tagore and Company. The country witnessed Raniganj coalfields as the major producer of coal for the entire 19th century and a major part of the 20th century (Chattopadhyay, 2001). But Coal mining activity badly affected the ecology, atmosphere, land, human health and water system viz. the surrounding environment of mining area. (Peplow and Edmonds, 2002; Younger, 2004). After completion of coal extraction, the pit is filled up by surface runoff and groundwater discharge. Then the pit becomes a water body or water reservoir (commonly called as Khadan) (Ghosh et al., 1984; Ghosh et al., 2005). Coal mining activity is a significant resource of soil and its heavy metal contamination (Liu et al., 2020).

The pit water body is mainly contaminated by the materials that are present in the adjoining top soil. The soil commonly contains toxic chemicals, pollutants, heavy metals etc. i.e. less nutrients for the growth of the angiosperms or any other plants (Ghose, 2001; Dutta and Agrawal, 2002; Johnson, 2003; Ghose, 2004; Razo et al., 2004; Pagnanelli et al., 2004; Marín-Guirao et al., 2005; Mercuri et al., 2005; Maiti, 2007; Bhuiyan et al., 2010; Sheoran et al., 2010; Das and Chakrapani, 2010). In Raniganj coalfield, the pit water contains high concentration of metals like Fe, Cu, Zn, Co, Cr, Mn, Pb, Cd, etc., leads to metal pollution in the coal pit ecosystem (De and Mitra, 2002). The pit-lake aquatic system depends on the physicochemical composition and nature of the bottom sediment and the growth and development of autotroph (like macrophytes) solely depend on the sediment of a water body that is called as the reservoir of nutrients (Barko and Smart, 1986).

Figure 1: Images of Four OCP



The availability of zooplankton from an OCP has lots of versatile bio indicating properties (Das and Chakrapani, 2011).These are: short life cycles, adaptation to changes of environment, vast distribution in various aquatic ecosystems, correlation in-between nutrient and energy cycles, and indicating to sudden environmental disturbances like acidification, thermal conditions, pollution, water flow and level of salinity, etc. (Litchman et al., 2013; Lokko et al., 2017; Pociecha et al., 2017; Zhao et al., 2018). The scarcity of water is a regular problem in Raniganj coalfield area which is supplied water by rain fed rivers - Damodar, Ajay and Barakar. This problem can be solved by of getting water stored in the open cast mine pits of this area and at the same time these water bodies can be used for pisciculture (Tiwary and Dhar, 1994). This present study aims at finding out how the accumulated heavy metals make an impact on fish diversity in different pits of two areas of Eastern Coalfield Limited in Raniganj coalfield in respect of different seasons.

MATERIAL AND METHODS

Study Area: The present study sites Ratibati & Damalia are now abandoned OCPs since 2017 and 1989 respectively, situated in Satgram area which is geographically around 23.674889°N Lat and 87.082754°E Long in Asansol sub div and Parasea & Ghanshyam OCPs both are abandoned since 2015-16 in Kajora area, located around 23.632407°N Lat and 87.171593°E Long in Durgapur sub div, Paschim Bardhaman. The Satgram and Kajora areas both are in E.C. Ltd. The Satgram area is surrounded by the Sripur Area and Kunustoria Area on the North, Kajora Area /Andal CD Block on the East, Bankura district on the South and Asansol sub div on the West. The Kajora area is confined by the Kenda Area on the North, Bankola Area on the East, Andal CD Block on the South, and Kunustoria Area on the West.

Figure 2: Satellite View of Four OCPs

The primary samples were collected from four study sites of three consecutive seasonal phases, these are pre-monsoon, post-monsoon seasons.

Table 1. Season Wise and OCP Wise Concentration Of Heavy Metal

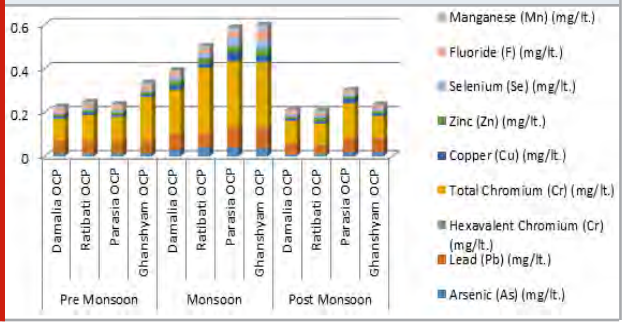
HEAVY METALS	Pre Monsoon				Monsoon				Post Monsoon				
	Damalia OCP	Ratibati OCP	Parasia OCP	Ghanshyam OCP	Damalia OCP	Ratibati OCP	Parasia OCP	Ghanshyam OCP	Damalia OCP	Ratibati OCP	Parasia OCP	Ghanshyam OCP	Permissible Limit as per MoEF Schedule-VI Standard
Arsenic (As) (mg/lt.)	0.015	0.014	0.014	0.015	0.03	0.04	0.04	0.036	0.01	0.011	0.02	0.02	0.2
Lead (Pb) (mg/lt.)	0.055	0.053	0.054	0.054	0.07	0.06	0.09	0.09	0.05	0.04	0.06	0.06	0.1
Hexavalent Chromium (Cr) (mg/lt.)	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.001	0.001	0.001	0.003	0.1
Total Chromium (Cr) (mg/lt.)	0.1	0.12	0.11	0.2	0.2	0.3	0.3	0.3	0.1	0.096	0.16	0.1	2
Copper (Cu) (mg/lt.)	0.01	0.011	0.01	0.01	0.02	0.02	0.04	0.03	0.01	0.01	0.02	0.01	3
Zinc (Zn) (mg/lt.)	0.01	0.011	0.01	0.01	0.02	0.025	0.03	0.03	0.01	0.018	0.01	0.012	5
Selenium (Se) (mg/lt.)	0.01	0.01	0.01	0.01	0.02	0.02	0.04	0.04	0.01	0.01	0.01	0.01	0.05
Fluoride (F) (mg/lt.)	0.015	0.016	0.015	0.014	0.02	0.021	0.025	0.04	0.01	0.01	0.01	0.01	2
Manganese (Mn) (mg/lt.)	0.01	0.011	0.013	0.02	0.01	0.013	0.019	0.03	0.01	0.01	0.011	0.01	2

After that these were brought to the laboratory for analysis by using APHA 23rd Edition, 1060. Parameters like As, Pb, Cr, Cu, Zn, Se, F, Mn were considered for observation. So many statistical methodologies were used in this study to explore the actual result. Arithmetic mean, ANOVA, frequency distribution (bar or comparative bar) was used. Standard protocols and methodologies were maintained during sampling and analyses of the mine water (BIS 1987). Whereas Correlation statistics was performed by using SPSS statistical software version 16.0 for analysing the data set and get better result. To know the seasonal nature of fish diversity in different abandoned OCP Shannon Weiner's Species Diversity Index (SDI) was applied. It is the most useful statistical tools to determine the species diversity in different time scale or in different area or condition. The formula used for Shannon Weiner's Species Diversity index:

$$H = -\sum_{i=1}^{s} pi \ln pi$$

Where, H = Shannon-Weiner species diversity index (SDI); $P_i = n_i/N$ (n_i = Number of individuals of i^{th} species and N= total number of individuals of all the species in the quadrate).

Figure 3: Showing season wise HM concentration in Four OCPs



RESULTS AND DISCUSSION

For this study, water samples were collected from 4 OCPs for heavy metals analysis, among which Damalia and Ratibati OCP fall under Satgram area of E.C. Ltd. and Parasea and Ghanshyam OCP fall under Kajora area of E.C. Ltd. Various types of heavy metals, i.e. As, F, Se, Zn, Cu, Cr, Pb, Mn were taken into consideration for the study. Due to high amount of heavy metals channelized through surface runoff in monsoon months, the water quality deteriorated in high scale.

Table 2. Two-way ANOVA for showing Season wise and OCP wise variation of significant levels of HM concentration

Source of Variation	ANOVA ON PRE MONSOON SEASON DATA					
	SS	df	MS	F	P-value	F crit
Between HM's	0.0475	7	0.0067	22.5226	1.35	2.7641
Between Seasons and OCPs	0.0006	2	0.0003	1.1553	0.343238	3.7388
Error	0.0042	14	0.0003			
Total	0.0524	23				
	ANOVA ON MONSOON SEASON DATA					
Source of Variation	SS	df	MS	F	P-value	F crit
Between HM's	0.1988	7	0.0284	571.4103	4.22	2.7641
Between Seasons and OCPs	0.0007	2	0.0003	7.4107	0.006381	3.7388
Error	0.0006	14	4.97			
Total	0.2002	23				
	ANOVA ON POST MONSOON SEASON DATA					
Source of Variation	SS	df	MS	F	P-value	F crit
Between HM's	0.0328	7	0.0046	27.0924	4.21	2.7641
Between Seasons and OCPs	0.0005	2	0.0002	1.4996	0.25	3.7388
Error	0.0024	14	0.0001			
Total	0.035763	23				

Notes: SS = Sum of Squares, df = Degree of Freedom, MS = Mean Sum of Squares, Fobs = Observed F, P- value = Probability, Fcrit = Critical F.

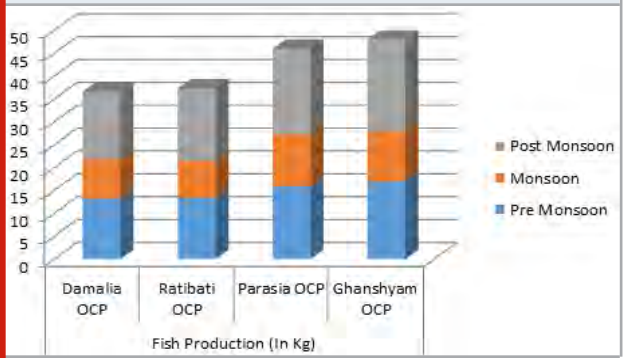
On the other hand, the rate of contamination decreased in pre-monsoon and it becomes the lowest in post-monsoon. So, it must be argued that post monsoon months are very favourable for aquatic ecosystem as well as fish breeding. Among the HM concentration, level of Chromium (Cr) and Lead (Pb) is very high in all season in respect of other

HM, but its level is maximum in monsoon months in all four OCPs. As and Se level were observed also high in respect other two seasons. Permissible limit as per MoEF (Ministry of Environment and Forest) schedule-VI standard is the scale of consideration for measuring the magnitude of HM concentration in different seasons.

Table 3. Season wise and OCP wise fish diversity (H)

SEASON WISE FISH CATCHING (Per net in kg)																
Scientific name	Local name	PRE-MONSOON					MONSOON					POST MONSOON				
		Damalia OCP	Ratibati OCP	Parasia OCP	Ghanshyam OCP	Fish Diversity (H)	Damalia OCP	Ratibati OCP	Parasia OCP	Ghanshyam OCP	Fish Diversity (H)	Damalia OCP	Ratibati OCP	Parasia OCP	Ghanshyam OCP	Fish Diversity (H)
Channa punctatus	Lata	0.3	0.2	0.5	0.4		0.2	0.2	0.5	0.3		0.3	0.3	0.6	0.5	
Clarias batrachus	Magur	0.3	0.3	0.4	0.3		0.3	0.2	0.4	0.2		0.3	0.4	0.5	0.5	
Oreochromis niloticus	Nilontica	0.2	0.2	0.2	0.3		0	0	0.2	0.2		0.2	0.3	0.4	0.6	
Labeo calbasu	Calbaus	2.1	2.3	2.3	2.5		1.5	1.3	1.7	1.5		2.3	2.5	2.6	2.8	
Catla catla	Catla	2.2	2.3	2.5	2.6		1.3	1.5	1.8	1.8		2.4	2.5	2.7	3	
Labeo rohita	Rui	2.8	2.8	3.1	3.4		2.1	2.2	2.3	2.1		2.9	2.9	3.5	3.4	
Labeo bata	Bata	0.7	0.6	0.9	1.1		0.5	0.4	0.7	0.8		0.9	0.9	1.2	1.6	
Hypophthalmichthys molitrix	Silver carp	1.1	1.1	1.4	1.8	2.31	0.8	0.7	1.1	1.2	2.28	1.3	1.4	1.8	2	2.37
Cirrhinus mrigala	Mrigel	1.3	1.3	1.7	1.6		0.5	0.6	0.9	0.8		1.5	1.5	1.9	1.8	
Aristichthys nobilis	Bighead Carp	0.4	0.3	0.5	0.5		0	0	0.3	0.4		0.5	0.6	0.6	0.8	
Cyprinus carpio	American Rui	0.8	0.8	1	1.1		0.4	0.5	0.5	0.5		1	1	1.2	1.1	
Ctenopharyngodon idella	Grass Carp	0.8	0.7	0.8	0.8		0.5	0.3	0.6	0.8		0.8	0.8	1	1	
Amblypharyngodon mola	Mourala	0.2	0.3	0.4	0.4		0.2	0.1	0.3	0.3		0.3	0.4	0.4	0.5	
Puntius sophore	Punti	0.1	0.2	0.2	0.2		0.1	0.1	0.2	0.1		0.2	0.3	0.2	0.4	

Figure 4: Season wise and OCP wise fish production (in kg)



The samples were collected during three different seasons and getting the impactful season in respect of heavy metal concentration in those OCP water and to find out the main controlling factor f it. It was found (Figure 3) that monsoon is the most vulnerable time for HM contamination.

Two-way ANOVA was also computed to analyse the significant level among HM's in different seasons and the significant variation in respect of season wise concentration of HM's in four OCPs. From pre-monsoon ANOVA (Table 2), it is said that there is significant difference in respect of different HM's ($F_{obs}>F_{crit}$) but among seasons and in terms of OCPs, there is no significant difference ($F_{obs}<F_{crit}$). From ANOVA on monsoon data regarding season wise and OCP wise HM's concentration (Table 2), it is shown that there is significant difference in terms of both cases, i.e. different HM's and Seasonal variation in OCPs ($F_{obs}>F_{crit}$). ANOVA on post-monsoon data (Table 2) represents the same condition like pre-monsoon time, like significant difference in respect of different HM's (Fobs>Fcrit) but among seasons and in terms of OCPs, there is no significant difference ($F_{obs}<F_{crit}$).

Rather, it is also found that the significance level is higher in all post-monsoon months. Season wise and OCP wise nature of fish diversity was also calculated using Shannon Weiner's Species Diversity Index, which is very well known index for measuring fish diversity in various aspects. More or less 14 fish species are found in these 4 OCPs during various seasons. These fishes have common names like rui, catla, calbaus, silver carp, punti, bata, mrigel etc. In all the OCPs, the most available fishes are rui, catla, calbasu, silver carp, mrigel. But their amount is varied during various seasons. Table 3

shows that the highest fish diversity (H) was found in post monsoon months, i.e. 2.37 and lowest diversity (H) seen in monsoon months, i.e. 2.28.

In respect of fish production (Figure 4), post-monsoon months are the highest productive months then other seasons. It is also showing that Parasea and Ghanshyam OCP of Kajora Area (ECL) are more productive than Damalia and Ratibati OCP of Satgram Area (ECL). OCP wise productions of fishes are varied as:

Figure 5: Fishing activity

Ghanshyam OCP>Parasea OCP>Ratibati OCP>Damalia OCP

West Bengal is a state covered by tropical monsoon season. Out of 3 seasons, i.e. Summer, Winter and Rainy season, Rainy or monsoon season is the dominant season. It comes after the long summer. During hot summer, huge amount of water vapour is released over the sky of Arabian sea and the Bay of Benagal.

Trade wind move towards land mass (impact of corriolis force) as the high pressure belts are prevailling at Indian land mass. Due to formation of densed cloud, exsessive amount of rainfall occurs throught the gangetic plain land. As a result of intensive rainfall, large volume of rain water flows as a surface runoff. These runoff water is contaminated with heavy metals, pollutants (both biotic and abiotic) etc. So the `inland water bodies also may be contaminated. In Raniganj area, OCPs are filled with this runoff water as well as ground water. But the water recharge basically happens during monsoon months through drainage and contaminated water. These OCPs are too deep in nature. Due to season wise variation of contamination, fish diversity is also varied. Top soil parameters are also the key factors to the nature of contamination. It may be stated that as the rainy season is the most affective time in terms of HM contamination, fish diversity and fish production is low at that time.

But due to the lowest rate of contamination in dry winter or post-monsoon months, high fish production and fish diversity are observed. On the other hand the HM concentration is low in the OCPs of Kajora Area than OCPs of Satgram Area. So fish production and fish diversity are higher in Parasea and Ghanshyam OCP than those in Damalia and Ratibati OCP. This observation is justified by Palit and Kar (2019) investigation that 15 most frequently naturally or cultured fish species occure under 4 orders, 5 families and 14 genera that were collected and identified from the OCP in Raniganj coalfield. After the analysis of OCP water quality and questionnaire survey of the local peoples, it is recomendated that pisciculture project can be initiated in 25 OCPs. OCPs aged more then 20 years turned naturally into wetland, harbouring a good amount of aquatic diversity, excellent quality of water without any restriction.

CONCLUSION

There are various future scopes regarding the optimum use of OCP water. It is observed that low nutrient level often minimises the fish foods and thus availability of primary production. It may be used for artificial or well managed fishing activities by the local people as sustainable developmental tools. For implementation of successful and sustainable fishing activity, pit lake fisheries need more considerations and attention on water quality, habitat and food sources which are the most important determinants. As these OCPs are too deep, trawl netting may be introduced for fishing.

Post-monsoon based planning may be initiated in that area as it is more productive and diversified season. Chemical water treatment through filtration may be introduced during rainy season. On the other hand, generally good water quality in OCP water contains elevated COPC which may bio magnify the OCP ecosystem and actually prevent a risk to end users and make profit for fisher men as well as local inhabitants. Proper training should be given to the local fisher men for advanced fishing techniques and procedure regarding OCP based fish production.

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Fungi Related with the Red Palm Weevil (Rhynchophorus ferrugineus) in the Hail Area, Northern Saudi Arabia

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ABSTRACT

Red palm weevil (RPW), a dangerous insect of date (Phoenix dactylifera), is an invasive insect not known to occur in the Arab region until recently. Control strategies for RPW include the utilization of pheromone traps and insecticides. The side effects of insecticides on people and the environment have driven researchers and policy makers to look for other methods of controlling RPW. As such, information on the normal adversaries of RPW and the defensive mechanisms of this insect against them is imperative to develop techniques for an integrated pest control. Conventional and molecular techniques were employed in the Hail region to identify and characterize the fungi associated with RPW to assess potential indigenous entomopathogenic fungi for biological management. Conventional identification methods indicated that the genera *Aspergillus* and *Fuzarium* spp were highly associated with RPW. *Aspergillus niger* contributed to the highest number of fungal isolates among all species, followed by *Aspergillus flavus* and *Fusarium solnum*. Other fungal isolates were tentatively identified as *Fusarium solani* (accession number: MH151017.1 with 100% sequence identity), *Fusarium proliferatum* (accession number: MK522076.1 with 100% sequence identity) and Nectria haematococca (accession number: DQ535183.1 with 99% sequence identity). Most of the isolated species in this study are saprophytic fungi which normally live in soil. However, some species are pathogenic fungi such some species of *Aspergillus* which would be potential candidates for biological control of RPW.

KEY WORDS: BIOLOGICAL CONTROL, DNA EXTRACTION, MYCELIUM, DEFENSIVE MECHANISM.

INTRODUCTION

The Kingdom of Saudi Arabia occupies an advanced position among the countries of the world in the cultivation of palm trees and the production of dates of all kinds, as the number of palm trees has reached 28.5 million, producing 1.3 million tons of dates annually. The date palm tree is an important part of the religious, cultural, and economic heritage of Semi-Arabian Island. This tree was exposed to grave problems that threatened the continuity of its production. The date palm tree is infected by many insect and fungal pathogens and pathogens, bacteria, nematode diseases, birds, weeds, and others organisms.

This insect is considered to be the most dangerous insect, where it feeds its larvae openly soft tissue inside the palm tree. This insect came from Southeast Asia to the Arab region and has been invading cultivated palms throughout the world. The red palm weevil, (RPW) causes

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considerable misfortune to date growers. In truth, it has been designated a most serious pest of date in Arab countries (Al-Shawaf et al., 2013). This insect belongs to the Curculionidae family in the order of Coleoptera (beetles and weevils). RPW is a hidden pest and remains inside the palm during the larval development and makes tunnels and then pupates. Subsequently, along with its omnipresent nature, these species are of incredible significance as they have effects on environment, agriculture, food production, biotechnology, and human and animal health (Abdel-Azeem et al. 2019 Qayyum et al 2020).

Biological control is a significant biological system administration and option in contrast boundless pesticide use (Crowder and Jabbour, 2014; Crowder et al., 2010). Biological control can harnessed as an environment administration which enhance hiding the pests from their adversaries such as predators, parasites and pathogens. This pest guideline by common enemies in crops offers natural and suitable conditions as harvest misfortune can be decreased because of over the top utilization of chemical pesticides (Bianchi et al., 2006).Numerous entomogenous fungi are generally normal, frequently inciting epizootics and consequently assaulting terrestrial insects in the groups Hyphomycetes and Entomophthorales. In contrast to other pathogens, the fungi entering the insect exoskeleton (Butt 2002).

The host can be tainted by (a) immediate treatment, (b) even transmission from contaminated insects or dead bodies to healthy insects, and (c) vertical transmission to resulting formative stages by means of the new age of spores. There are several studies which have been conducted to investigate the capability of fungi against insect pests. *Beauveriabassiana* and *Metarhiziumanisopliae* are among the most common entomopathogenic fungi which have been utilized in the control of insects throughout the world. Impressive research have been done to utilize entomopathogenic parasites as biocontrol agents against RPW (Sewefy et al., 2009; Demibilo et al, 2010; Guerri-Agullo et al, 2010 Belein 2018 Qayyum et al 2020). However, such information is lacking in Saudi Arabia, therefore, the purpose of this study was to identify and characterize the fungi associated with RPW, invading date palm trees in Hail province. Understanding the connection between fungi and insects should be helpful in the improvement of biocontrol agents utilizing entomolopathogenic fungi as an elective control system for this genuine insect pest.

MATERIAL AND METHODS

Sampling: One hundred and thirty adults of RPW were gathered from 5 infested farms in the study area (Hail area) during the period of October 2019 to May 2020. These were Al gayed, Jubbah, Helala, Horir and Guthasharagiya, and all of the collecting sites occur within latitude 27.523647 and longitude 41.696632. A pheromone trap was utilized for the collection of RPW. 30 males and 30 females were placed in plastic boxes, then maintained in a freezer at –20 °C at the microbiology laboratory until used for investigating the associated fungi.

Isolation and preliminary identification of fungi:The infected RPW adults were utilized to start stock colonies, and the colonies were periodically supplemented with the introduction of additional wild specimens. Infected plants were assessed for the presence of RPW by visual observation and listening for the feeding sounds the insect by touching the ear to the stem of the palm. Fungal growth was induced by placing the RPW in moisture chambers at 20-22 °C for 24-48 hours, then recovering the fungi by direct and indirect isolation techniques (Zho et al., 2008).

For direct isolation of fungi, small amounts of the visible hyphal mat were inoculated onto a Petri dish containing potato dextrose agar. While for isolating fungi using indirect method specimens were surface sterilized, then dissected, placed in Petri dishes containing PDA and were incubated at 25°C. The presence of growing fungal colonies was observed every day. The PDA plates were checked daily for fungal growth. After 5-10 days of incubation, several fungal colonies were grown on the plates. The mycelium of each fungal colony was transferred and maintained on fresh PDA plates. For identification of fungi, a small part of pure fungi colony was cut from the colony edge using a sterilized scalpel. The fungi plugs were placed in Petri dishes. The fungal isolates were identified to the genus level based on microscopic features (morphology of hyphae, fruiting bodies, gamic spores, and conidia) and macroscopic features (colony morphology, colour, and growth rate) using the appropriate identification keys.

Frequency of occurrence: Existence of fungi collected from the RPW samples was determined utilizing the following formula: F= (NF/NT) X 100% Where: F = Frequency of occurrence % NT= total number of samples

B– DNA Extraction: The method modified by Dellaporta et al. (1983) was utilized for the DNA extraction as outlined below:Twenty 20 mg of fresh harvested mycelium were ground with a pestle in a 1.5 ml tube, and 33 µl of 20% sodium dodecyl sulfate (SDS, w/v) and 160 µl of 5 M potassium acetate KoAc (Sigma chemicals) were added and vortexed. Then the mixture was centrifuged for 10 min at 10,000 rpm, and 450 µl of supernatant was transferred to a new tube. 450 µl of phenol, chloroform, and isoamyl-alcohol (PCI) were added with a ratio of 25:24:1 and vortexed, then centrifuged for 5 min at 10,000 rpm. 400 µl of the upper phase was then removed to a clean microcent7. The supernatant was removed and the total nucleic acid was precipitated in the bottom of the tube. The pellet were washed with 70% ethanol and spun 5 min at 10,000 rpm. Then the pellet was re-suspended in 100 µl of Double-distilled water (ddH_2O).

C– Polymerase Chain Reaction (PCR): For fungal isolates, Two primer pairs, the forward IT5 primer (5'-GGAAGTAAAAGTCGTAACAAGG-3') and the reverse

ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the entire ITS region (White et al., 1990). The purified PCR products were sequenced by Macrogen Inc., (Korea), and sequencing of the purified isolates was performed in both directions using ITS5 and ITS4 primer pairs. Sequence alignments were edited by MEGA7 (Kumar et al., 2016).

RESULTS AND DISCUSSION

Frequency and identification of fungi associated with the red palm weevil: Fifty-six fungal isolates were successfully isolated from the bodies of RPW (Table 1 and figure 1& 2). Using conventional methods, 38 fungi that were identified to the genus level, which incorporated several members of the genera *Fusarium* and *Aspergillus*. Among all the isolates the most dominant fungal species was *Aspergillus niger* (17 isolates), followed by *Aspergillus flavus* (11 isolates), *Fusarium* sp. 1 (4 isolates), *Fusarium* sp. 2 (3 isolates), *Aspergillus* sp. 1 (1 isolate), *Aspergillus* sp. 2 (1 isolate), and *Aspergillus* sp. 3 (1 isolate). In addition, 18 isolates could not be identified.

Table 1. Occurrence of fungal species from RPW in various sites

Fungal species	No. of isolates	Prevalence (%)*
Fusarium sp. 1	4	7.1
Fusarium sp. 2	3	5.3
Aspergillus niger	17	30.35
Aspergillus flavus	11	19.6
Aspergillus sp. 1	1	1.7
Aspergillus sp. 2	1	1.7
Aspergillus sp. 3	1	1.7
Unidentified sp. 1	8	14.28
Unidentified sp. 2	4	7.1
Unidentified sp. 3	3	5.3
Unidentified sp. 4	1	1.7
Unidentified sp. 5	1	1.7
Unidentified sp. 6	1	1.7

*Prevalence was calculated based on the total number of fungal isolates (N/56)

Molecular identification of the fungi: The results of using molecular identification showed that of the fungal isolates were tentatively identified as *Fusarium solani* (accession number: MH151017.1 with 100% sequence identity), *Fusarium proliferatum* (accession number: MK522076.1 with 100% sequence identity) and *Nectria haematococca* (accession number: DQ535183.1 with 99% sequence identity) as indicated in Table 2 and Figure 1.

According to the World Health Organization WHO (2013), there are approximately 23-25 million people infected every year with pesticide toxins, and nearly 20,000 of them die annually (Schmutterer, 2002). Therefore, there was a need to develop non-toxic, safe, human and animal alternatives as one of its control methods intended to reduce the population of the pests or its harmful effects, to the extent that it does not constitute with severe harm to humans, animals, or their activities. This can be achieved by means of other biological species from insects, nematodes, bacteria, fungi, etc. Pathogenic fungi to insects are one of the important factors due to their wide presence in nature in addition to being invisible, expensive and highly specialized for treating specific pests (Shiff; 2014., Rajesh et. al., 2002).

Figure 1: Frequency of fungi isolated from the bodies of the red palm weevil.

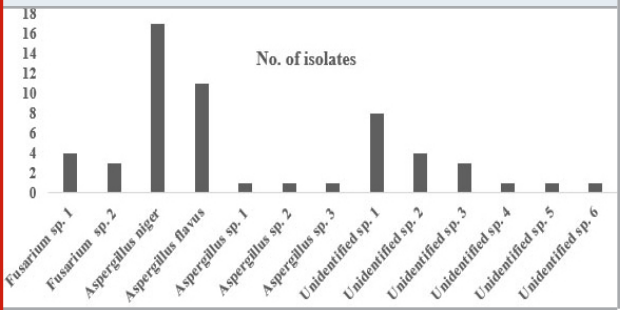
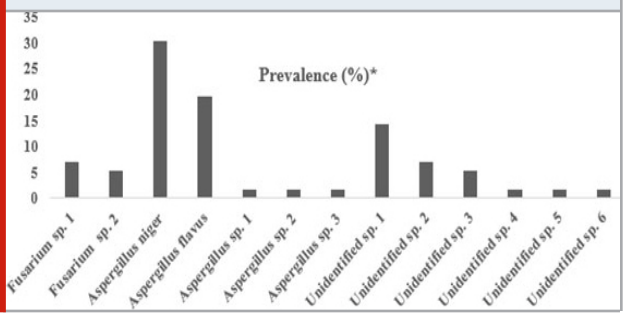


Figure 2: Frequency of fungi Prevalence % from the bodies of the red palm weevil.
Note. Prevalence was calculated based on the total number of fungal isolates (N/56)



The outcomes of the present study indicated that the most frequently encountered fungal species associated with RPW collected from various farms in the Hail region was *Aspergillus spp*., specifically *Aspergillus niger* and *Aspergillus flavus* as identified with conventional identification methods. *Aspergillus niger* constituted 30.35%, whereas *Aspergillus flavus* constituted 19.6% of the total number of fungal isolates. Furthermore, *Fusarium* sp. 1 and Fusrium sp. 2 were identified with a percentage 7.1% and 5.3%, respectively. On the other hand, 31.78% of the fungal isolates were unidentified using conventional methods. Using molecular biological techniques, the dominant fungal species were *Fusarium solani*, *Fusarium proliferatum*, *Fusarium proliferatum* and *Nectria haematococca*, respectively. *Aspergillus* spp. and *Fuzarium* spp. were highly associated with the RPW. *Aspergillus niger* contributed to the highest number of fungal isolates among all species. This suggests that *A. niger* lives as saprotroph or symbiont with RPW.

Surprisingly, fungi considered as saprotrophs or symbionts just assimilate the organic matter from the host, yet they don't have capacity to taint the host (Dube 2007). A few different fungal species, which have low number of fungal isolates (e.g., *Aspergillus flavus*, *Fusarium solani* and *Fusarium proliferatum*) were likewise found as saprotrophs on soil, spices, insects, and plants. This shows that RPW have an extraordinary relationship with soil and plant fungi. This could be attributed to the inclination of insects to contact the ground or plant-related fungi. Other than that, the strategy utilized for getting the fungi may most likely mirrored the aftereffects of this study. The thorough review of fungi associated with RPW could be acquired by utilizing distinctive isolation strategies coupled with culture-dependent techniques, which will uncover a few species other than the saprophytic ones.

Table 1. Basics characteristics of school children

Sp.	Taxon	SGB	ID%
1.	*Fusarium proliferatum*. (Matsush.) Nirenberg	MK522076.1	100
2.	*Nectria haematococca*. Berk. & Broome	DQ535183.1	99
3.	*Fusarium solani*. (Mart.) Sacc.	MH151017.1	100

It is known that saprophytic fungi are the largest group of macro fungi, responsible for breaking down and recycling dead plant and animal material. Therefore, the presence of these fungi is not surprising since saprophytic fungi are generally recognized from the fruiting bodies observed on dead trees, leaf litter, animal bones, even feces. *Saprophytic fungi* discharge enzymes to separate and digest the lignin, cellulose, or chitin in this material into simple soluble aggravates, that can be consumed by them, and by plants, as supplements. In this manner, they assume an imperative role in diminishing the collection of dead natural material and in reusing fundamental supplements, especially carbon and nitrogen (https://fungimap.org.au/about-fungi/saprophytic-fungi/).

Several species of the fungus Aspergillus are considered biological control agents for many insects, these species included: *Aspergillus fumigatus*. *Aspergillus nidulans* and *Aspergillus flavus* which infect the larvae and adults of honey bees, causing the disease (Brood stone). However, the success of infection depends on the inheritance of the fungus, its growth rate and capacity to exploit the insect's materials for growth, as well as its ability to produce dermal-degrading enzymes, poisons, and overcoming insect resistance (Cole and Rolinson 1972). *Aspergillus niger* fungus is one of the most common types of *Aspergillus*. It causes diseases to fruits and vegetables called black rot. The black spores of *A. niger* evidently protects against sunlight and UV irradiation, prompting an upper hand over different microorganisms in their living spaces.

These capacities guarantee its progressively presence in warm habitat (Krijgsheld et. al., 2012). Some scientists tend to argue that some strains of *Aspergillus niger* produce toxins called okratoxin. However, this is not always the case, as some dispute this claim by saying that the previous reports are the result of a misdiagnosis of the fungi species. Recently, evidence has been obtained that strains of *Aspergillus niger* actually produce octeratoxin (Schuster et. al., 2002).

Aspergillus flavus, the second fungus associated with RPW in the present study, a well-known species related with aflatoxin contamination of agricultural crops (Cotty et al. 1994, Cotty 1997). It was also found that the fungus *A. flavus*. had an effect on silkworms, as this fungus secreted type 1 aflatoxin (Raper et. al., 1965). The following metabolites in fungus A. Parasiticus may have a role in killing the insect (aflatoxin toxins G2, G1, B2, B1) as well as secreting the following enzymes: Aminopeptidase, N-acetyl-β-glcosaminindase, Alkaline protease, Serineprotenase. The genus *Fusarium* is a filamentous fungi, widespread in nature, as it is found in soil is in the decaying organic matter of plants and animals as well as food residues, and many types of waste.

The genus presently contains almost under 200 accepted species, and its monetary and authentic significance causes it to stay at center stage in future conversations about terminology and mycological variety. Subsequently, along with its omnipresent nature, these species are of incredible significant effects on environments, agriculture, food production, biotechnology, and human and animal health (Abdel-Azeem et al. 2019). *Fusarium* sp. cause many plant diseases such as root rot and vascular wilt. Also, species of this genus are associated with animals such as nematodes, spiders, amphibians and reptiles. Some species were documented as insect parasites and an insect pathogen. Roberts (1981) stated that this fungus has the effect of acting as pesticides against different insects.

Fusarium solani is likewise connected with opportunistic infections of humans and other animals, causing systemic infections with a high death rate, as well as restricted infections in the skin and other body parts (Gupta et. al., 2000). *Fusarium solani* is considered ubiquitous, while the distribution of some other species depends more on climate conditions (Summerell et al. 2010).Preceding host intrusion, certain fungal attributes assign them harmful or destructive organism. The organism must produce high number of conidia with strong adhesion that allow them to enter into the host through straight forward penetrating structures. Besides, the attacking organism must have the ability to sidestep or beat the host immune system by creating toxins. In future, experiments must be

directed to investigate in detail the immune mechanism of RPW that may assist with discovering genes engaged in host defense (Abid et. al., 2013).

Naturally occurring bio-control agents are a desirable choice to switch the utilization of dangerous synthetic insecticides. Among these microbes, the utilization of entomopathogenic fungi was discovered to be promising substitute for insect control. As per a gauge, in excess of 700 species of fungi having a place with various genera, are known to infect insects. Before, the capability of entomopathogenic fungi particularly *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* have been assessed against various pests including *Aphis craccivora* (Saranya et. Al., 2010), *Aedes aegypti* (García-Munguía et. al., 2011), *Bemisia argentifolii* (James et al., 2003), *Coptotermes formosanus* Shiraki (Hussain el al., 2010), *Melanoplus sanguinipes* (Inglis et al., 1996), *Ocinara varians* Walker (Hussain et. al. 2009), *Odontotermes obesus* (Hussain et al. 2011), *Periplaneta americana* (Mohan et.,al., 1999), *Rhynchophorus ferrugineus* (Dembilio et. al., 2010), *Scolytus scolytus* (Doberski 1981), *Thrips tabaci* (Al-mazra'awi et al 2009).

The accomplishment of these naturally occurring microorganisms principally relies upon the host pathogen interaction.The entrance of entomopathogenic fungi to attack the host is through the cuticle that involves complex biochemical interactions between the host and the pathogen (fungus) before germination, penetration, growth, and reproduction of the fungus. Preceding the host attack, there are certain characteristics of fungi that assign them destructive or a virulent strains (Abid et al 2013).

CONCLUSION

In summary, the successful control of RPW depends largely upon interactions between the host and its pathogens. There is a steady battle among host and a pathogen that eventually leads to the success or failure of the latter. In case of compatible interactions, the pathogen must have strong adhesion capacity that eventually penetrates into the host. *Aspergillus* app and *Fusarium* spp. dominated fungal flora of RPW. These fungi might have effects in management of this destructive pest. Most of the isolated fungal species in this study are saprophytic fungi which normally occur in soil. Thus, some species are pathogenic fungi such as *Aspergillus* spp. which would be potential candidate for biological control of RPW.

Authors' Contribution: All authors contributed in designing, carrying out, and reporting this research.

Competing Interest: All the authors declare that they have no competing interest that can affect the current study.

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Induction of Defense Proteins in Tomato Treated with Streptomyces griseus Against *Fusarium oxysporum* f. sp. *Lycopersici*

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ABSTRACT

Plants have endogenous defense mechanisms that can be induced in response to attacking insects and pathogens. Inducing the plant's own defense mechanisms by prior application of biological inducer is thought to be a novel plant protection strategy. Synthesis and accumulation of PR proteins have been reported to play an important role in plant defense. By considering this, the study was undertaken to assess defense enzymes of PP pathway by *S. griseus* against challenge inoculation with *Fusarium oxysporum f. sp. Lycopersici* (FOL). The *S. griseus* formulations were evaluated for their competence in regulating wilt disease and growth promotion of tomato under greenhouse circumstances. Further, induction of defense proteins against challenge inoculation with FOL in tomato plants were also studied. In the present research, *S. griseus* has been introduced in to the root system of tomato plants well in advance of Fusarium oxysporum infestation. A noteworthy reduction in disease severity (19.5%) and improved yield (520.0 g/plant) also significant increase in plant growth over control was observed in tomato plant treated with chitin amended S. griseus (T3; root dipping) against FOL. β - 1, 3 glucanase and chitinase were persuaded to accumulate at elevated level on 15th day of challenge immunization in T3 plants. Correspondingly leaves of *S. griseus* (T3; root dipping) treated plants expressed higher activity of peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) after a day and it reached maximum on 15th day of inoculation with FOL. Equally, phenolics, chlorophyll and carbohydrates were found to a mass in bacterized (chitin amended *S. griseus*) tomato leaf tissues challenged with FOL and reached extreme on 15th day of pathogen *S. griseus* inoculation. These outcomes put forward that defense enzymes tangled in phenylpropanoid pathway was induced by *S. griseus* might have contributed the restriction of invasion of *F. oxysporum* in tomato leads to plant protection from wilt disease facilitated by *Fusarium oxysporum f. sp. lycopersici.*

KEY WORDS: WILT DISEASE TOMATO, S. GRISEUS TREATMENT, DISEASE REDUCTION.

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INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most significant commercial vegetable crop grown in India. Tomato roots/stem is affected by highly destructive soil borne pathogen *Fusarium oxysporum f. sp*. lycopersici (FOL), leads to leaf wilting, yellowing, substantial loss in yield and eventually plant death (McGovern, 2015; Bubici, 2018). The features of soil-borne pathogen (i.e., invading through the vascular tissue) make it difficult to control the disease and biological control agents emerge to hold promise in pathogen management (Ben Abdallah et al., 2016). Plant growth promoting rhizobacteria (PGPR) are being exploited commercially for plant protection via colonizes at rhizosphere, plant immunization or induce systemic resistance against pathogens (Dahal et al., 2017).

The plant responses involved, expression of increasing level of numerous pathogenesis associated proteins (PR proteins) comprises (a) β-1, 3 glucanases (PR-2 family), chitinase (PR-3 family) which lysis the fungal cell wall; defense gene products including (b) Peroxidases (PO), Polyphenoloxidases (PPO) with the intention of catalyze lignin formation (c) *Phenylalanine ammonia*-lyase (PAL) concerned in phytoalexins and phenolics synthesis (Zouari et al, 2016; Mhlongo et al, 2018). Activated induced resistance (via ISR or SAR) is a broad-spectrum and long-term resistance, which usually suppresses a disease up to 20–85% (Abbasi et al., 2019).

Actinomycetes, particularly *streptomyces* sp., encompass a far above the ground potential to control fungal pathogens since it produces antifungal antibiotics, proteins and cell wall degrading enzymes. They may live saprophytically and endophytically in agricultural environments where they colonize the rhizosphere in addition different parts of plant (Saleem et al., 2016). *Streptomyces* sp., have been reported as PGP and biocontrol agent against *Verticillium dahliae* (Cao et al., 2016), *Phytophthora drechsleri* (Sadeghi et al., 2017), *Fusarium oxysporum* (Abbasi et al., 2019, Hussein and Al-Dulaimi, 2020) *Phytophthora capsici* (Abbasi et al., 2020). There is no considerable learning on various defense enzymes of PP pathway and PR proteins owing to *Streptomyces griseus* treatment. Herewith, the study was undertaken to assess the induction of defense enzymes of PP pathway and accumulation of PR-proteins by *S. griseus* against challenge inoculation with *Fusarium oxysporum f. sp. Lycopersici* (FOL).

MATERIAL AND METHODS

Plant material: Tomato variety Co-4 susceptible to wilt disease was analysed in this study.

Isolation of pathogen: The naturally infected tomato plants evidenced for wilt disease, situated at Nachipalayam Village, Coimbatore were collected in sterile polyethylene bags. Diseased stem and root tissues were sliced (1 – 1.5 cm), inoculated in Potato Dextrose Agar plates (PDA) and incubated at 28 ^{0}C for 5 – 7 days. The hyphal tips raised from the segment was purified by single spore isolation method, identified as *Fusarium oxysporum* (FOL) and maintained in PDA slants at 4^{0}C until further use (Aileen, 2006).

Pathogenic fungal inoculum: The respective isolates of FOL, was multiplied in sand maize medium (Riker and Riker, 1936). Approximately 2 x 108 cfu/g of inoculum of FOL in sand maize medium was mixed with sterilized soil at 5% (W/W), then infested into greenhouse earthen pots ten days prior to transplanting 45 days old seedling (Larena et al., 2003).

Bacterial isolate: *Streptomyces* sp., was isolated from prawn cultivated pond soil of Peddapuram Village; East Godavari District using Colloidal Chitin Agar (CCA) plates, identified as *Streptomyces griseus* (Acc. No. 9723) and deposited in MTCC, Chandigarh. The culture was maintained in actinomyces agar slants at 4^{0}C till further use.

Development of bioformulation of *S. griseus*: Talc-based bioformulation of *S. griseus* (Acc. No. 9723) with (i) chitin, (ii) without chitin, (iii) self-fused S. griseus, (iv) chitinase enzyme of S. griseus with Apsa 80 (carrier molecule) were developed as described by Anitha and Rabeeth, (2009). The prepared formulations were filled in polythene bags, sealed and kept back at room temperature for greenhouse studies.

Efficacy of *S. griseus* against fusarium wilt of tomato under greenhouse conditions: The formulations were evaluated for their competence in regulating wilt disease of tomato under greenhouse circumstances. The treatments were imposed as seed treatment, seedling dip and foliar applications.

Seed bacterization: Seeds of cultivar Co–4 were surface sterilized, soaked in water containing talc-based *S. griseus* (with or without chitin)–10 g/kg of seeds (Meena et al., 2002) for 12 hrs. At the end, the seeds were drained off, dried under shade for overnight and sown in (27 x 42 x 7 cm^3) trays containing vermiculite and sand [1:1; (V:V)] ratio (Vidhyasekaran et al., 1997).

Seeding dip and foliar spray: For root inoculation, surface sterilized seeds of cultivar Co-4 were sown in nursery trays mentioned above. After 45 days, seedlings were pulled out from the trays and roots were immersed in chitin amended S. grises and self-fused *S. griseus* (20 g/L) formulations for 30 mins before transplanting the seedlings. Whereas chitinase of *S. griseus* along Apsa 80 was prearranged as foliar spray to transplanted seedling.

Efficacy of *S. griseus* on disease severity and experimental design: An experimental design comprises, T_1 – plants raised from seeds treated with talc formulation of *S. griseus* (10 g/kg of seeds), T_2 – plants raised from seeds treated with chitin amended talc formulation of *S. griseus* (10 g/kg of seeds), T_3 – plant roots immersed in chitin amended talc formulation (20 g/L) of *S. griseus*,

T_4 – plant roots immersed in talc formulation (20 g/L) of self-fused *S. griseus*, T_5– foliar spray using crude chitinase enzyme (1L) of *S. griseus* with Apsa 80 (113.3 IU/mL) after planting, T_6– plants raised from carbendazim treated seeds (2 g/kg of seeds), served as chemical check, T_7– plants neither treated with bacterial suspension nor challenged by the pathogen (Healthy Control) and T_8– plants challenged with pathogen (Inoculated Control).

After the treatments, the seedlings were transplanted into greenhouse pots at the rate of four seedlings/pot which is artificially infested with pathogen (Sand: Maize Medium). Ten days after transplanting, foliar spray near the roots of treated plants were given as per (T1 to T6) and irrigation was given consequently. Later 15 days, external symptoms of wilting and yellowing of leaves (Fusarium wilt) were recorded. The Disease Severity (DS) was calculated as Disease Severity (DS) = Number of leaves with symptoms / Total number of leaves X 100 (Pascale et al., 1999).

Three replications were maintained in each treatment; each replicate comprises of six pots and in each pot four plants were maintained. The relative humidity in the glasshouse was maintained around 80%, and the temperature of 26^{0}C (day) and 20^{0}C (night). The experiments were laid out with randomized block design with four replications and the experiments were repeated once under greenhouse bench.

Efficacy of *S. griseus* on plant growth promotion: After 45 days, two plantlets from each replication were sampled and the efficacy of diverse treatments on root & shoot length, fresh & dry weight of root and shoot, yield of the plant was recorded in all the treatments challenged with FOL in comparison with control.

Efficacy of *S. griseus* on induction of defense related enzymes: Leaf samples were collected at different time intervals (0, 5, 10, 15 and 20 days) after challenged with FOL pathogen. Three plants were sampled from each replication of the treatment separately (mentioned in experimental design) and maintained for biochemical analysis. Freshly collected leaf samples (0.1g) were washed with sterile distilled water and homogenized in 2 mL of ice-cold phosphate buffer (0.1 M; pH 7.0) at 4°C. The homogenate was centrifuged at 10000 rpm at 4°C for 15 min, the 181 collected supernatant was stored at –20°C until used for biochemical assays. Assay of accumulations of PR proteins and induction of defense enzymes of PP pathway like chitinase (Wen et al., 2002), β-1, 3-glucanase (El–Katatny et al., 2000), peroxidase (PO) (Hammerschmidt et al., 1982)., polyphenol oxidase (PPO) (Mayer et al., 1965), phenylalanine ammonium lyase (PAL) (Dickerson et al., 1984), total phenol (Zieslin and Ben–Zaken, 1993), total chlorophyll and total carbohydrate (Sadasivam and Manickam, 1991) were measured.

Statistical analysis: The data's were statistically analyzed independently (Gomez and Gomez, 1984). The treatments mean was compared by Duncan's Multiple Range Test (DMRT) at 5% significant using SPSS software version 7.0.

RESULTS AND DISCUSSION

Plants have endogenous defense mechanisms that can be induced in response to pathogen attack. The defense genes are considered to be inducible and ideal stimuli or signals are preferable. It is considered a novel plant protection strategy to incorporate the plant own defensive mechanisms through biological inducers (Bubici et al., 2019). By considering the above the effectiveness of prior application of *S. griseus* in controlling the soil-borne diseases has been documented.Talc based formulations of S. griseus with / without chitin supplements were prepared and analyzed against FOL under greenhouse conditions. Talc formulation of P. fluorescens (Pf1) and *B. subtilis* against rust and leaf spots of groundnut (Meena et al., 2002), sheath blight infection of rice (Radja et al., 2002) and fruit rot of chilli (Bharathi et al., 2004) have been studied.

In this line, talk formulation of *Streptomyces corchorusii* against rice (Tamreihao et al., 2016) *Streptomyces rochei* against tomato (Zamoum et al., 2017) has been reported as effective against disease management. The host plant provides the habitat and nutrients necessary for the pathogen to become established at the infection site. *Fusarium oxysporum* is a soil-borne pathogen and can spread through root system and it would be better to protect the infection sites rather than alter the entire soil microbial community. Root application of the biocontrol agents has been approved in the literature (Shishido et al., 2005; Yigit and Dikilitas, 2007, Bibusi et al., 2019).

Henceforth, in the present research, *S. griseus* has been introduced in to the root system of tomato plants well in advance of Fusarium oxysporum infestation. A noteworthy reduction in disease severity (19.5%) and improved yield (520.0 g/plant) over control was observed in tomato plant treated with chitin amended *S. griseus* (T3; root dipping) against FOL. Whereas in inoculated control (T8), there was uppermost disease severity (61.1%) with negligible yield of 120.0 g/plant was observed (Table 1). Chemical (carbendazim) treatment had less effect compared to *S. griseus* treated plants but then the activity was overwhelmed the healthy control (T7). The prior applicant of S. griseus in root system prevents the pathogen entry that resulted in significant reduction in disease severity.

Equally, *S. enissocaesilis* and *S. rochei* (Abbasi et al., 2019), *Streptomyces* sp. and *Pseudomonas fluorescence* (Hussein and Al-Dulaimi, 2020) lightened the fusarium wilt of tomato (FWT) disease symptoms like chlorosis, stunting and wilting better than carbendazim. Recently, Wu et al., (2019) have reported that a decreased percentage disease index of rice sheath blight caused by *Rhizoctonia solani* was associated with an increase in streptomyces sp., derived antifungal agent concentration. In contrast, *Streptomyces* sp., treated banana plantlets (root dipping) showed significant ($P<0.05$) reduction

in wilt severity (47%) compared to untreated plantlets was reported by Getha et al., (2005) and Bubici et al., (2019).

The plant growth was significantly influenced by applying *S. griseus* at root tissues. Compared with inoculated control (T8), noteworthy development was perceived in T_3 plants followed by T_4 plants. Though, the sensible growth was observed in carbendazim treated plants (T6), it failed to provide appreciable growth than the *S. griseus* (T_3) treated plants (Table 2). The outcomes indicated that the S. griseus may endorse the growth directly by producing plant growth regulators / stimulating nutrient uptake / synthesizing antibiotics in contradiction of pathogens. Correspondingly, inoculation of *S. enissocaesilis* and *S. rochei* (Abbasi et al., 2019), actinobacteria (Passari et al., 2019), *G. fasiculatum* (Anusha and Krishnaraj, 2017) and *G. intraradices* (Akkopru and Demir, 2005) has encouraged the growth and yield of tomato plant against FOL inoculation was predicted in earlier. *Likewise, Streptomyces corchorusii* treated rice plants showed significant increase ($P \leq 0.05$) in root and shoot lengths, fresh root and shoot, and dry shoot weights over the control was reported by Tamreihao et al., (2016).

Current exploration implies that prior application of *S. griseus* strengthen the host cell wall resulting in restriction of pathogen invasion in plant tissue (Abdul et al., 2020). In base line, synthesis and accumulation of PR proteins have been reported to play an important role in plant defense (Abbasi et al., 2019; Balint-Kurti, 2019). However, there is a little information available on defense responses induced by *S. griseus* in plants against pathogen invasion. The present study clearly figured that the induction of enzymes involved in phenylpropanoid metabolism and accumulation of PR-proteins in tomato leaf tissue have been encouraged by chitin amended *S. griseus* (T3; root dipping) in response to challenge inoculation with *F. oxysporum f. sp. lycopersici* over the inoculated control (T8).

Induction of β-1,3 glucanase and chitinase enzymes play a key role against fungal attack in turn directly connected with the structural relevance of chitin and glucans in the cell wall of fungi (Bubici, 2018). These enzymes act upon the fungal cell wall resulting in loss of inner contents of cells and eventually leads to death. In our study, β-1, 3-glucanase (412.09 µmol/h/g) and chitinase activity (475.25 µmol/h/g) in self fusant *S. griseus* (T_4; root dipping) treated plants was found to be lower than the plants treated chitin amended *S. griseus* (T_3; root dipping) (β-1, 3-glucanase of 424.25 µmol/h/g; chitinase of 492.0 µmol/h/g). Significant activities were observed on 15 days after inoculation (Fig. 1 & 2) for the treated plants, whereas in inoculated control (T8) plants both enzyme activities were renowned at ground level.

Hence, initiation of enzymes has been occupied in defense against further invasion of the pathogen in root through degrading its physical barrier. In general, accumulation of hydrolytic enzymes is more pronounced in the biocontrol agent's pretreated plants compared with inoculated control. Similarly, Chandrasekaran and Chun, (2016) and Magesh and Devi (2017) have attributed that induced resistances in tomato, in correlation with an increased activity of chitinase, β-1,3 glucanase and 1-4 glucosidase.

Figure 1: Induction β-1, 3 glucanase by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.

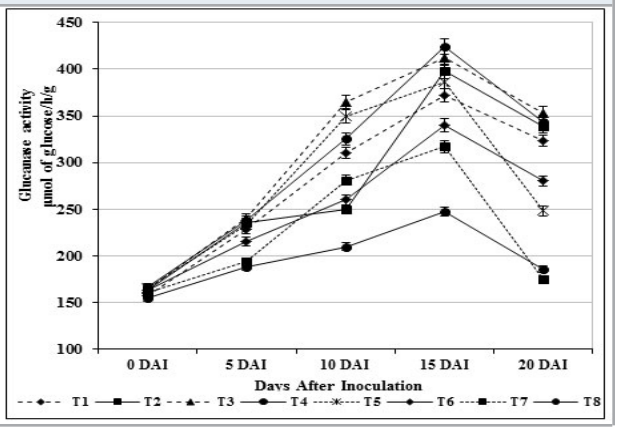
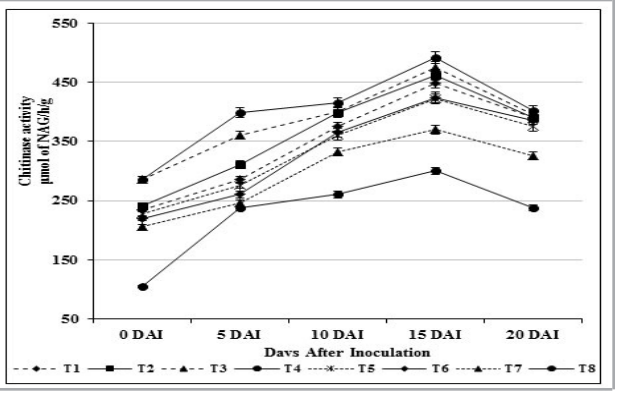


Figure 2: Induction chitinase enzyme by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications



PO is a key enzyme, has been implicated in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross linking, IAA oxidation, cross linking of extension monomers and oxidation of hydrosyl-cinnamyl alcohol into free radical intermediates with wound healing (Magesh and Devi, 2017). The plant resistance phenomenon is associated with biosynthesis of lignin in which PO is playing a key role (Liu et al., 2018). PO activity was significantly increased in T_3 plants on 15 days after inoculation with FOL (4.93 absorbance/min/g) whereas the activity was insignificant in inoculated control (T_8) (2.23 absorbance/min/g). PO activity in the leaves of carbendazim treated plants (T_6) did not elucidate any remarkable changes throughout the experimental period (Fig. 3). This unique PO induced by *S. griseus* isolate might have contributed to induce defense in

tomato root tissue against FOL invasion. In accordance with the above an increased peroxidase activity in *P. fluorescens, Streptomyces* sp., and *S. rochei* treated plants challenged with *Fusarium oxysporum* – a wilt pathogen was addressed earlier (Nikoo et al., 2014; Salla et al., 2016; Mahesh and Devi, 2016; Abbasi et al., 2019).

Figure 3: Changes in peroxidase activity by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.

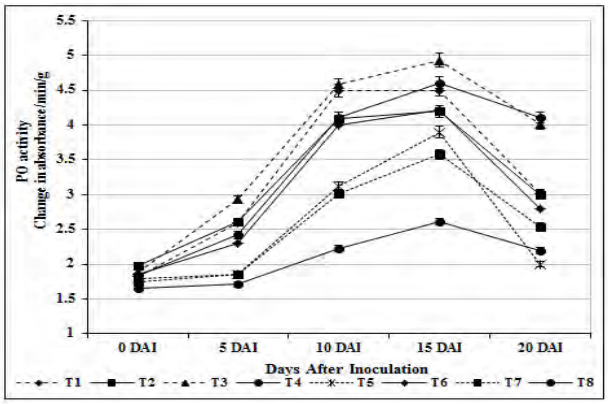
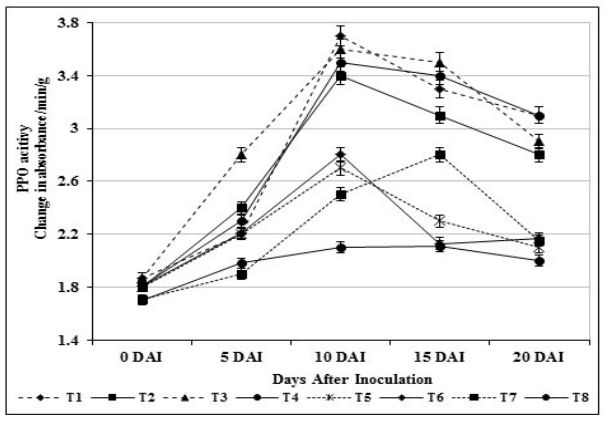
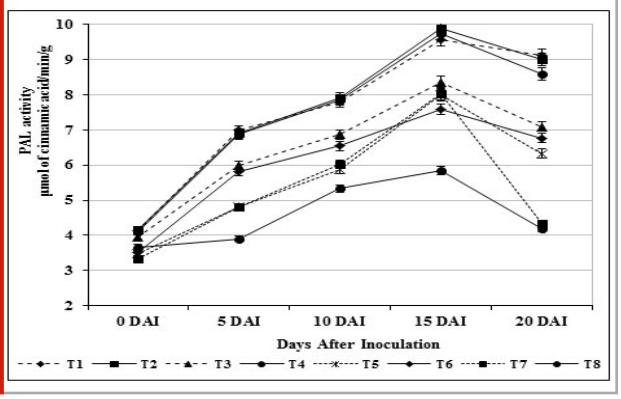


Figure 4: Changes in polyphenol oxidase activity by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.



PPO is an important defense related enzyme produced under stress condition and accumulates upon wounding in plants. Correspondingly, the expression of PPO in T_3 plants were conferred to be higher when compared with other treatments (T_1 to T_7) and inoculated control (T8) plants (Fig. 4). The PPO activity in inoculated control plants (T_8) was 2.1 absorbance/min/g were as chitin amended S. griseus (T_3; root dipping) showed 3.5 absorbance/min/g 15 days after inoculation. In connection with this, carbendazim treated plants (T6) exhibited adequate activity compared to inoculated control (T_8) and healthy control (T_7) plants but the activity was less compared to T_3 plants. The higher expression of PPO might have implicated in induced defense responses against the pathogen invasion. Similarly, higher accumulation PPO in Bacillus subtilis (Chandrasekaran and Chun, 2016) *P. fluorescens* treated tomato plants (Magesh and Devi, 2017; Ramamoorthy et al., 2002) in response to *Fusarium oxysporum* induced wilt disease was reported earlier.

Figure 5: Accumulation of phenylalanine ammonium lyase activity by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.



PAL played an important role in the biosynthesis of phenolics and phytolexins (Sharma et al., 2019). It catalyzes the deamination of L-phenylalanine to trans cinnamic acid, which is the first step in biosynthesis of large class of plant natural products based on the phenylpropane skeleton including lignin monomers as well as phytolexins. Experimental results revealed that chitin amended *S. griseus* (T_3) treated plants showed highest activity (9.75 µmol/min/g) of PAL. There was no marked change in carbendazim treatment (T_6) during the time course of experimental period and the accumulation of PAL remained higher compared to the untreated control (T_8) and healthy control (T_7) (Fig. 5). Increased activity of PAL due to *S. griseus* isolate treatment might have prevented the fungal invasion, and thus, the activity maintained at higher levels. Besides, *B. subtilis*, *Streptomyces* sp. and *S. rochei* treated tomato roots rendered higher PAL activity when compared to untreated control was propounded by Chandrasekaran and Chun, (2016); Magesh and Devi, (2017) and Abbasi et al., (2019).

Phenols are fungi toxic in nature and higher accumulation played an important role in plant resistant by enhancing the mechanical strength of host cell wall and by inhibiting the fungal growth (Aoun, 2017). The outcome of the study would demonstrated that quantitative changes in phenolics (0.66 mg catechol/g) were improved in tomato seedlings exposed to chitin supplemented *S. griseus* (T_3) during the experiment against pathogenic *F. oxysporum* over control indicated that, the response is truly systemic and physiological state of the plant has been altered (Fig. 6). Perhaps the findings of Loganathan et al., (2014) and Panina et al., (2007) were recorded maximum phenol aggregation in various biocontrol agents treated plants. The increased phenolic substances

exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content of pathogen was reported by Benhamou et al., (2000).

Figure 6: Accumulation of phenols by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.

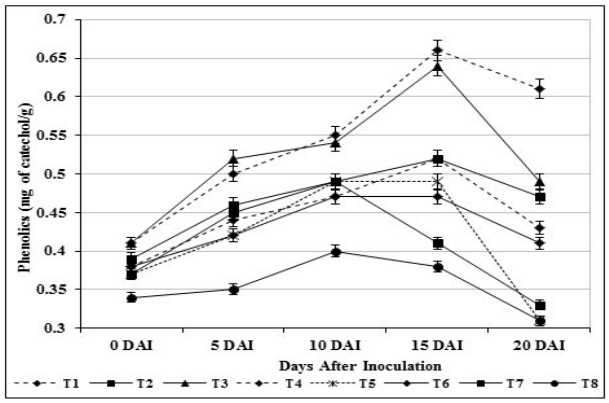
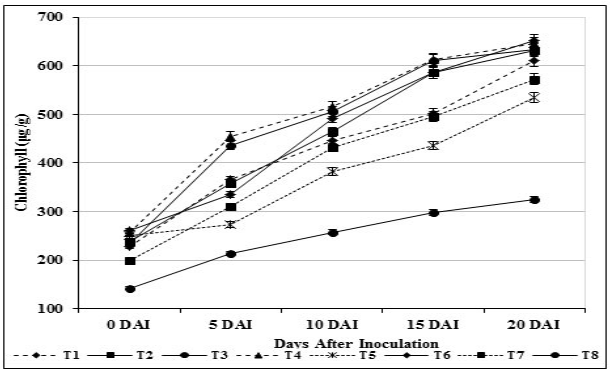


Figure 7: Accumulation of chlorophyll by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.



The chlorophyll content in inoculated control plants (T_8) was found to be 324 µg/g, which was lower (652 µg/g) than carbendazim treated plants (T_6) and other treatments. Total carbohydrate content (564 µg/g) in leaf tissues of treated (T_4) tomato plants was found and it was observed as high compared with inoculated control (T_8) (Fig. 7). Likewise, 556 µg /g of carbohydrate was observed on 20th day in T_3 plants which was found to be higher than the carbendazim treated plants (453 µg/g) and healthy control (T_7). The findings specified that the photosynthetic area was confined which consecutively produced higher amount of carbohydrates (Fig. 8). This finding is somewhat similar to results reported by Dias et al., (2017) and Passari et al., (2019) indicating that *Streptomyces* sp. isolate PM5 and *Streptomyces thermocarboxydus* produced a greater amount of chlorophyll a & b than control plants. Babu et al., (2015) also reported that the rhizobacteria *B. subtilis* and *Azotobacter chroococcum* induced higher levels of chlorophyll content in tomato plants, relative to control plants. In contrast, Kandan, (2000) have stated lower sugars leaves in leaves of resistant cultivar.

Figure 8: Accumulation of carbohydrates by *S. griseus* in tomato plants challenged with the pathogen *F. oxysporum f. sp. lycopersici*. Vertical bars indicate standard deviations of three replications.

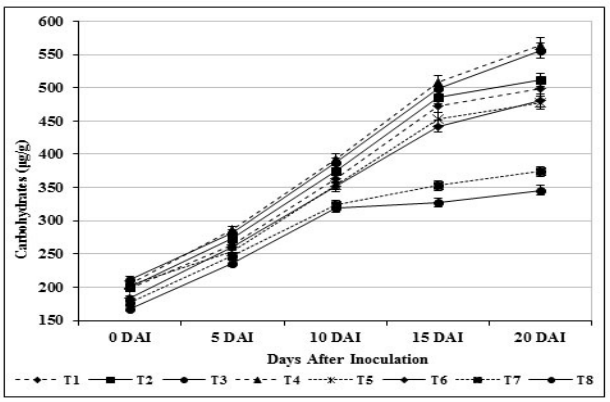


Table 1. Efficacy of *S. griseus* on disease severity and yield of tomato against *Fusarium oxysporum f. sp. lycopersici*. Values are the mean of triplicates. In a column, means followed by a common letter are not significantly different at 5% level by DMRT.

Treatments	Disease severity (%)	Yield (g/plant)
T_1 – *S. griseus* (Seed treatment – 10 g / kg)	26.0[a]	485.0
T_2 – Chitin amended *S. griseus* (Seed treatment – 10 g/ kg)	23.1[a]	455.0
T_3 – Chitin amended *S. griseus* suspension (Root dipping – 4 x 108 cfu/mL)	19.1[a]	550.0
T_4 – Self fusant *S. griseus* suspension (Root dipping - 4 x 108 cfu/mL)	19.5[a]	520.0
T_5 – Foliar spray using crude chitinase enzyme (1L) of *S. griseus* with Apsa 80 (113.3 IU/mL) after planting	28.9[ab]	416.0
T_6 – carbendazim treated seeds (2 g/kg of seeds)	31.5[ab]	425.0
T_7 – Healthy Control	49.3[de]	425.0
T_8 – Inoculated Control	61.1[h]	120.0

Under greenhouse conditions, seeds treated with *S. griseus* (T_1 and T_2) and the plants treated with crude chitinase enzyme of *S. griseus* with Apsa 80 (T_5; foliar

spray - 113.3 IU/mL) did not produced significant amount of defense enzyme and PR protein against wilt disease of tomato plant incited by *Fusarium oxysporum* than the plants treated with *S. griseus* (root dipping) (T_3 and T_4). But then relatively significant effect (defense proteins) was experienced in comparison with carbendazim treated (T6) and inoculated control plants (T8).

From this investigation it is clear that *S. griseus* used along with chitin has been introduced to tomato root systems (root dipping) performed well in the management of FOL collectively appreciable yield and plant growth. The interactions between plant and pathogen have stimulated defensive enzymes initially but later, as the pathogen colonizes the root tissue, defensive enzymes dropped dramatically. At present, higher accumulation of enzymes of phenylpropanoid metabolism and PR-proteins has been instigated in tomato treated with *S. griseus* (T_3 – root dipping) in response to invasion by *F. oxysporum f. sp. lycopersici*. In conclusion, biocontrol efficiency could be accomplished by introducing the *S. griseus* in to the root system well in advance of pathogen infestation evidenced for significant colonization of *S. griseus* which controls the invasion of fungi and diminishes the pathogen density which simultaneously triggered the plant-mediated defense mechanism might have collectively contributed to induced resistance against FOL. Hence, the contemporary investigation recommended that chitinolytic *S. griseus* could be used as a promising biocontrol agent in Integrated wilt disease management.

Table 2. Efficacy of *S. griseus* on growth promotion of tomato against *Fusarium oxysporum f. sp. lycopersici*. Values are the mean of triplicates. In a column, means followed by a common letter are not significantly different at 5% level by DMRT.

Treatments	Length (cm)	Shoot Fresh wt. (g)	Dry wt. (g)	Length (cm)	Root Fresh wt. (g)	Dry wt. (g)
Treatment 1	43.9^{ef}	22.8^{e}	6.3^{fg}	31.6^{gh}	14.5^{f}	2.5^{d}
Treatment 2	45.7^{fg}	23.6^{f}	6.5^{gh}	32.5^{gh}	14.8^{g}	2.6^{de}
Treatment 3	47.5^{g}	25.8^{h}	6.8^{gh}	32.7^{gh}	15.8^{h}	3.9^{fg}
Treatment 4	50.6^{h}	24.7^{g}	6.7^{h}	33.0^{h}	15.6^{b}	3.8^{gh}
Treatment 5	38.4^{d}	21.8^{de}	5.8^{de}	30.6^{gh}	13.9^{ef}	2.4^{ed}
Treatment 6	40.4^{de}	20.8^{ef}	5.2^{b}	29.9^{g}	13.9^{e}	2.2^{ab}
Treatment 7	34.7^{bc}	19.9^{c}	4.5^{a}	22.7^{a}	13.9^{b}	1.9^{a}
Treatment 8	26.9^{a}	15.9^{a}	5.8^{b}	20.4^{de}	9.8^{a}	1.7^{a}

Conflicts of Interest: Authors declare that they have no conflicts of interest.

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Isolation and Characterization of Alkaline Protease Producing Streptomyces tendae SO-13 from Rhizosphere Soils of Western Ghats

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ABSTRACT

Microbial alkaline proteases are among the salient hydrolytic enzyme and used extensively as biocatalysts, Extracellular alkaline protease is of great importance due to its applications in detergent, food and leather industries by the prokaryotic actinomycetes. The present investigations were focusing on soil actinomycetes. Screening for alkaline protease and characterization of specific strain from rhizosphere soil from Western Ghats of Karnataka, India. The zones of hydrolysis were observed on skim milk agar medium under pH 10.0 at 30±02 0C for 5 to 7 days and produced 117.67 U/mL of alkaline protease by the fermentation. Morphological and biochemical characterization of the isolate was carried out and found that the isolate belongs to the *Streptomyces* genus. Further species confirmation of the *Streptomyces* was done by 16S rRNA gene sequencing. The obtained nucleotide sequences SO-13 were submitted to the GenBank database and the accession number assigned is MW130237. The results reveal the isolate SO-13 was identified as *Streptomyces tendae*. The present data reveal that the isolate SO-13 represents *Streptomyces tendae*. The potentiality of the eco-friendly enzyme could be used for various industrial applications. The alkaline protease production from *Streptomyces tendae* SO-13 is the potent strain for commercial use. Further investigation at the commercial level and novel applications of alkaline proteases to be carried out.

KEY WORDS: WESTERN GHATS, RHIZOSPHERE SOIL, STREPTOMYCES TENDAE, ALKALINE PROTEASE.

INTRODUCTION

The Western Ghats of India has been considered as one of thirty-four biodiversity hot spots in the world, with rich flora and fauna. The Western Ghats of Karnataka popularly known as Sahyadri hills are treasure houses of endangered species but less studied concerning microbial biodiversity. Actinomycetes are widely distributed in soil, and constitute a significant part of soil microflora (Bawazir and Manjula 2018). Soil actinomycetes are prokaryotes with various metabolic activities (Chavan et al., 2013). Actinomycetes are gram-positive, filamentous, bacteria, characterized by the formation of aerial mycelium, and spores on solid media, with DNA high in G+C content of 60-70 mol% (Shirling and Gottlieb 1966; Subbaraju and Onkarappa 2018).

Among the microorganisms, actinomycetes acquire special importance as the power source of antibiotics and, other bioactive primary and secondary metabolites such as enzymes (Chavan et al., 2013). Sources of commercial enzymes cover a wide range from microorganisms then animals and plants sources. Fungi and yeast contribute about 50%, bacteria 25%, animal 8% and plant 4% of the total in commercial enzymes production (Azmi et al., 1999). Proteases of neutrophilic as well as alkaliphilic

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bacterial and fungal origins are utilized for commercial exploitation (Pathak and Rothad 2018). Several microbial species are reported for the alkaline protease productions. The most copiously studied the alkaline protease producers among bacteria are the genera of *Bacillus* (Keshavamurthy et al., 2018) and *Pseudomonas* (Alexander et al., 2012), Streptomyces species (Sarkar and Suthindhiran 2020).

Alkaline proteases are also utilized for therapeutic agent's development. The oral administration of proteases from *Aspergillus oryzae* to aid in digestion and rectify lytic enzyme deficiency syndromes is already in practice (Mikawlrang, 2016). More recently, actinomycetes are an auspicious source of a wide range of important enzymes. *Streptomyces* protease preparations that are profit-oriented used include FRADIASE 7M *(S. fradiae*) and PRONASE 7M (S. griseus). While alkaline proteases from bacteria are vastly characterized, similar attention has not been paid to actinobacteria. Currently, antibiotics are the major bioactive compounds from the actinobacteria. However, in these prokaryotes, the ability to produce a variety of enzymes may be an attractive phenomenon (Govindharaj et al., 2016). In the present investigation, the new isolate SO-13 capable of producing the alkaline protease. The study includes morphological, biochemical and molecular identifications.

MATERIAL AND METHODS

Isolation Proteolytic actinomycetes from soil samples: The rhizosphere soil samples were collected in a sterile Ziplock plastic cover from a depth of 10-15 cm from the Western Ghats in Karnataka, India (Around the latitude 140 13l 07.3ll N and longitude 740 49l 57.2ll E). The samples were air-dried for 6 to 7 days and ground in a mortar using a pestle (Lingakumar et al., 2014; Subbaraju and Onkarappa, 2018). The samples were serially diluted and 0.1 ml of 10^{-2} to 10^{-6} dilutions were plating on Starch Casein agar medium (g/ml: Starch 10; Casein 3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4$ 0.05; $CaCl_2$ 0.02; $FeSO_4.7H_2O$, 0.01; agar 20, distilled water 1000ml pH=7.0± 0.2) described by Guravaiah (2016). The plates were incubated at 30±20 ^{0}C for 5 to 7 days. The isolates were grown on starch casein nitrate agar medium at 30±20 ^{0}C and stored at 4^{0}C for short term storage.

Screening for alkaline proteolytic activity: Proteolytic activity from isolated pure cultures was screened by plating on Skim milk agar Supplement with Na_2CO_3 for detection alkaline protease producing isolate (g/L Skim milk, 100.0; Yeast exact, 5.0: Na_2CO_3, 10.0; Agar, 20; distilled water, Final pH=10). Incubate for 5 to 7 days at 30±02 ^{0}C (Davoudi et al., 2014).

Characterization of potent alkaline producing isolate SO-13: The classifications of actinomycetes were originally based upon the morphological observations. Preferably, the *Streptomyces* species were identified and recorded using the Bergey's Manual of Systematic Bacteriology. Primarily, the characterizations of actinomycetes isolates were done by their colony morphology, spore colour, aerial mass colour and substrate mycelium, pigmentation appearance on the medium. The isolate was subjected to grams and acid-fast staining procedures. The isolate was identified to genus level based on their spore chain arrangement by coverslip technique and spore surface ornamentation by SEM analysis (Gautham et al., 2012).

Biochemical tests of isolate: Standard biochemical tests were employed, the Indole test, Methyl red test, Voges – Proskauer test, Citrate utilization test, Urease test, Catalase test, Degradation of Cellulose, hydrolysis of Casein, Gelatin and Starch to determine the potent alkaline protease producing Strain (Shirling and Gottlieb 1966; Gautham et al., 2012; Subbaraju and Onkarappa 2018).

16S rRNA gene sequencing of the isolate: The strain was further subjected for identification by 16S rRNA sequence using universal primers and genomic DNA as a template. The genomic DNA extracted from the isolate by using spin column kit (HiMedia, India). Bacterial 16S rRNA gene (1500 bp), (Clarridge JE, 2004) was amplified using polymerase chain reaction (PCR) in a thermal cycler and were purified using Exonuclease I -Shrimp Alkaline Phosphatase (Exo-SAP) (Darby et al., 2005). Purified amplicons were sequenced by Sanger method in ABI 3500xL genetic analyzer (Life Technologies, USA). Sequencing files (.ab1) edited using CHROMASLITE (version 1.5) and further analyzed by Basic Local Alignment Search Tool (BLAST) with closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences (Altschul et al., 1990). A duly annotated partial nucleotide sequence of the strain was deposited with NCBI Genbank (https://www.ncbi.nlm.nih.gov). Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software to construct the Phylogenetic tree using the neighbour-joining method (Tamura et al., 2011).

Alkaline protease production and enzyme assay: Alkaline Protease production by the selected Strain SO-06 was carried out by One ml of fresh isolate inoculum were added 100 ml of production medium (Glucose,10.0g/L; casein, 5.0 g/L; yeast extract, 5.0 g/L; $K_2H\ PO_4$, 2.0 g/L; KH_2PO_4, 2.0 g/L; $MgSO_4$. 7H2O, 1.0 g/L and at pH 9.0-9.5) into 250ml Erlenmeyer flask. The flask was placed in a rotary shaker incubator at 150 rpm at 30 °C for 5 to 7 days. The fermentation broth was centrifuged at 8,000rpm for 20 min at 4 ^{0}C to obtain the crude culture filtrate (Hosseini et al., 2016). The alkaline protease activity of the crude enzyme was done by taking 0.5 ml of culture filtrate was added to 0.5 ml of 1% casein (a substrate) in 0.1 M Phosphate buffer (pH 7.0) and then incubated for 10 min at room temperature.

To stop reaction 3ml of 10% (w/v) trichloroacetic acid and the mixture was centrifuged at 5000 rpm for 10 min. The supernatant, 5 ml 0.5M $Na2CO_3$ solution and 0.5ml of two Folin Ciocalteau reagent was added and mixed thoroughly, incubated for 30 min at room temperature in

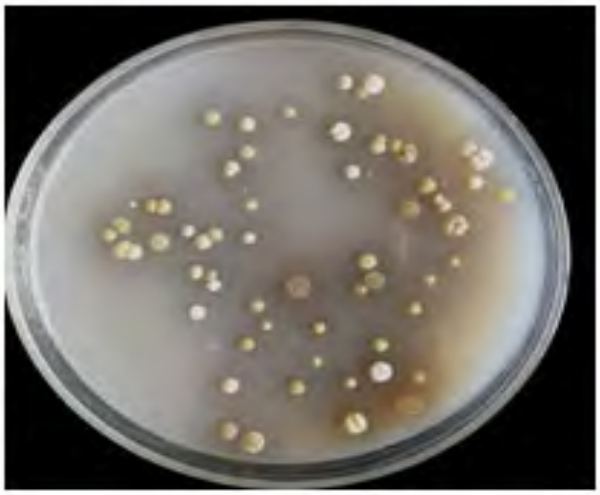
dark condition. The optical density of blue colouration was measured using the UV-VIS spectrophotometer at 660 nm and the blank (Keshavamurthy et al., 2018). The amount of the released amino acids was calculated using the tyrosine standard. One unit of enzyme activity represents the amount of the enzyme required to release 1µg of tyrosine per ml per min (Pant et al., 2015).

RESULTS AND DISCUSSION

Fifteen rhizosphere soil samples were collected in sterile Zip lock plastic cover from at different Latitude and longitude of the Western Ghats in Karnataka, India (Figure 1) and air-dried soil samples were subject to isolation and based on the zone of hydrolysis on agar plates, SO-01 to SO-16 visible colonies were obtained on 10^{-2} to 10^{-6} dilution Petri plates (Figure 2).

Figure 1: The map showing sampling stop of Western Ghats, Karnataka.

Figure 2: Visible colonies on 10^{-4} dilution petriplate



Isolate SO-13 was found to have alkaline protease activity depend on diameter zone of hydrolysis of proteolytic on the plate (Figure 3). Similar result found in *Streptomyces griseorubens* E44G (Rashadb et al., 2015) and *Streptomyces Indus* (Guravaiah 2016) for proteolytic activity. Hence the isolate SO-13 was selected for further studied.

Characterization of the potent alkaline protease producing isolate SO-13: The result showed a diverse morphological characteristic with spore colour-grey, colony morphology-radial, substrate-white and aerial mycelium-grey (Figure 3). The spore-bearing hyphae and spore chains show straight-flexuous with open and spore surface Ornamentation-smooth (Figure 4). Isolate shown the gram-positive, filamentous, rod structure, identified by gram staining and Nonacid fast. The biochemical characteristics as potent alkaline protease isolate SO-13 exhibit positive for methyl red, voges-proskauer, citrate, urease, catalase, hydrolysis of casein, starch and gelatin (Table 1). Based on the spore chain arrangements the isolate was assigned to the genus *Streptomyces* sp. Similar results were observed in *Streptomyces* sp. (Takeuchi et al., 1996) and *Streptomyces tendae* AR1 (Laidi et al.,2006).

Figure 3: Zone of hydrolysis by the isolate *Streptomyces tendae* SO-13

Figure 4: SEM of the isolate *Streptomyces tendae* SO-13

The 16S rRNA gene was amplified through PCR which showed 1500 kb band on 2% agarose gel (Figure 5). Subsequently, 16S rRNA gene sequence analyses were carried out to elucidate the taxonomic relationships among closely related *Streptomyces* species. The strain

SO-13 has been matriculated into a cluster containing *Strepomyces tendae*. Based on the phylogenetic analysis the strain was closely related to *Streptomyces tendae* strain ATCC 19812 exhibiting high similarity (99.88 %) (Takeuchi et al., 1996). So it is assigned as *Streptonyces tendae* SO-13 (Figure 6). Similar results were noticed in *Streptonyces tendae* AR1 (Laidi et al., 2006) and *Streptonyces tendae* 1 & 2 (Bahamdain et al., 2020). The obtained nucleotide sequences SO-13 were submitted to the GenBank database and the accession number assigned is MW130237. The results reveal the isolate SO-13 was identified as *Streptomyces tendae*.

Table 1. Morphological and Biochemical Characterization of isolate *Streptomyces tendae* SO-13

Morphological identification	
Media	Starch Casein Nitrate
Growth	Abundant
Colony Morphology	Radiating
Aerial Mycelium	Grey
Substrate Mycelium	White
Diffusible Pigment	No
Spore Arrangement	Straight-flexuous
Spore surface Ornamentation	Smooth
Biochemical tests	
Indole	-
Methyl Red	+
Voges – Proskauer	+
Citrate	+
Urease	+
Catalase	+
Casein	+++
Starch	++
Cellulose	-
Gelatin	+ +
pH	7.0-10.0
NaCl	5%
Tentative genera	*Streptomyces* sp.

Streptomyces tendae SO-13 was subjected to secondary screening for quantitative protease production. The protease produces 117.67 U/mL during 5 to 7days at pH 9.0. Similar results were observed in *Saccharomonospora viridis* SJ-21 (Hosseini et al., 2016) and *Streptomyces flavogriseus* HS1 (Sofiane et al., 2014). *Streptomyces* were industrially important organisms for commercial production of Protease. Alkaline proteases have been produced from numerous *Streptomyces* like thermoalkaline proteases by marine *Streptomyces* sp. D1 (Madanrao et al., 2013). *Streptomyces*. sp LL-DAP (Karthik et al., 2010) and *Streptomyces flavogriseus* HS1 (Sofiane et al., 2014) are also reported for protease production.

Figure 5: Agarose gel electrophoresis of *Streptomyces tendae* SO-13
L-Step-up 1 Kb DNA Ladder
Lane 1- 16S rRNA amplicon

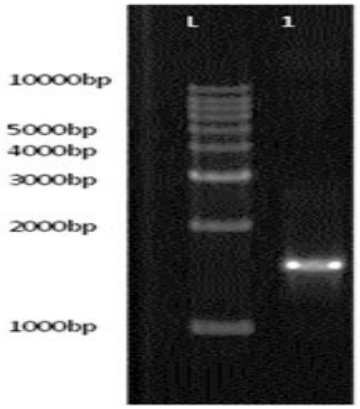
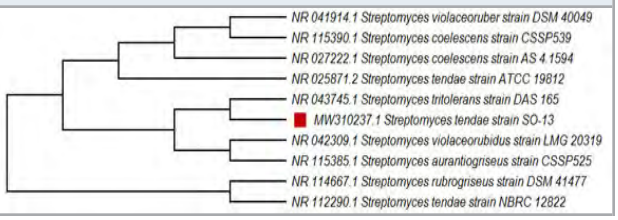


Figure 6: Phylogram obtained based on phylogenetic analysis of 16S rRNA gene sequence data showing the phylogenetic positions of isolate *Streptomyces tendae* SO-13



CONCLUSION

The Western Ghats are the treasure house for novel actinomycetes species associated with the rhizosphere soil. The characterizations of alkaline protease *Streptomyces tendae* SO-13 was confirmed by morphology, biochemical and 16S rRNA sequencing. The alkaline protease producing from *Streptomyces tendae* SO-13 is the potent strain for commercial production. Further investigation at the commercial level and novel applications of alkaline proteases to be carried out.

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Patients' Perception Towards Receiving Dental Treatment from Undergraduate Students of a Saudi Dental School.

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ABSTRACT

The aim of the present study was to investigate the factors responsible for the patients attendance at undergraduate dental clinics, the degree of satisfaction patients have after the treatment is done and the amount of trust the patients have towards their treating dental students. The cross sectional qualitative survey study was conducted among male and female patients, attending the College of Dentistry, King Saud University Hospital, Riyadh Saudi Arabia, at the undergraduate training institute from August 2019 to February 2020. The patients were randomly selected who were attending hospital for dental treatments in undergraduate course with 3rd, 4th and 5th year students. The questionnaire consisted of 17 closed ended questions mainly divided into section one for demographic data and section two for undergraduate clinics method of booking, type of procedures, method of contacting the student clinic and previous experience with student clinics. The last section enquired about participants beliefs related to student clinics, including, satisfaction level and reasons for dissatisfaction, would they prefer repeating the experience, must students be supervised and are students able to manage emergencies. Data was analyzed Descriptive statistics (means, standard deviations, percentages and frequencies), One-way analysis of variance (ANOVA), post hoc tukey and Chi square test to compare association between categorical variables. 268 out of 400 responses were completed (67%). 67.2% of participants were males, while 32.8% were females. 42.9% were aged between 18-28 years and 23.1% were aged between 29-39 years, however, 30.2% of participants were students and 27.6% were governmental employees. In addition, 38.1% had bachelor's degrees while 29.1% completed secondary; 43.3% were single, 52.6% were married. Two-thirds of the patients were satisfied with the dental treatments provided by students under supervision and the main reason for dissatisfaction was the delay and long duration of treatment. A majority agreed that they would seek treatment from dental students again and one-third of patients believed that students do not need supervision at all times. Further studies with larger sample size and different undergraduate institutes should be performed to validate these findings.

KEY WORDS: PATIENTS' PERCEPTION, METHOD OF CONTACTING, DESCRIPTIVE STATISTICS.

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INTRODUCTION

Dental students are trained and prepared to perform the clinical procedures by taking courses in their undergraduate years delivered by experienced faculty members on clinics and laboratory simulations (Alrahabi et al., 2015). In addition, exposure to basic life support and cardiopulmonary resuscitation courses, where students are trained to manage life-threatening emergencies is

mandatory for students (Al-Shamiri et al., 2017, Alotaibi et al., 2016). Furthermore, another critical aspect of undergraduate teaching and learning is infection control management and introduction to proper protective measures to eliminate risks of transmission of infections to patients and the surrounding environment. Ethics and professionalism are perilous foundation skills for a dental graduate and are taught through didactic and practical interactions at educational institutes.

It is critical to understand that all required competencies for suitability of graduation of any student including examining, diagnosing and treating patients clinically, are taught under close supervision of experienced and highly trained faculty instructors. Therefore, in the undergraduate years of dentistry, dental students are required to perform clinical requirements and achieve competency in all dental specialties to qualify for graduation. In order to achieve such objective, it is crucial to provide a students with patients who are willing to be treated by dental students. This somewhat could be challenging taking into consideration students' lack of expertise and chance of errors, (Humphris et al., 2002, O'Keefe et al., 2014, Gilmouret al., 2016,Al-Essa, 2017 Al-Harbi et al., 2019).

One of the main determinants of a successful physician-patient relationship is trust establishment (Hall et al., 2001). It is not between doctors and patients only, but it also should be remained between all the workers at any place (Berthelsen et al., 2010, Firth-Cozens 2004, Carter 2009). Patients are no longer classified as a passive recipient (Shikiar & Rentz 2004). Various initiatives that supports patients' encouragement have been established to activate patient's role at all levels (Shikiar & Rentz 2004). In addition, patients can uniquely contribute in detecting problems of quality and safety in health care systems (Vincent 2002, Gillespie & Reader 2018).

Moreover, absence of patient's trust in dentistry as well as in medicine can construct a barrier to seeking health-care which may result in poor patient satisfaction, lack of compliance, increase in anxiety and thus can lead to unfavorable outcomes (Yamalik 2005). It is linked with factors including empathy, reliability, responsiveness, assurance, accessibility, cost and more (Davies & Ware 1981, Tucker & Adams, 2001).Patient satisfaction towards the dental treatment provided by the undergraduate students at dental schools is multifactorial and is of great importance for the growth and evolvement of oral health service and practice, (Scambler & Asimakopoulou, 2014).

Objection and complaints made by patients may cause a great deal of anxiety and stress among the students and their supervisors (Humpris et al., 2002). Evaluating patients' acceptance and compliance towards the dental care they receive from dental student is of great importance for the success of an oral health service can be assessed by the amount of satisfaction of its patients and positive feedback (Scambler & Asimakopoulou, 2014, Essa et al., 2006). In this regard, some patient still strongly believe that public hospital are failing in delivering quality services (Aman et al., 2016). A recent study conducted at the College of Dentistry, King Saud University, showed that the patients were satisfied with their treatment provided by trained students and maintained a good oral hygiene. However only 86 participated in the study (Albiladi et al., 2019).

Also, various studies around the world described patient satisfaction towards dental and medical treatment in general ,but still studies regarding patient feedback after receiving dental treatment from undergraduate students is limited (Deshwal et al., 2014). Therefore the aim of this study was to investigate the factors responsible for the patient's attendance at under-graduate dental clinics, the degree of satisfaction patients have after the treatment is done and the amount of trust the patients have towards their treating dental students.

MATERIAL AND METHODS

The study protocol was approved by the ethics review board at the College of Dentistry, King Saud University, Riyadh KSA and is presented using standard guidelines. The cross sectional qualitative survey study was conducted among male and female patients, attending the Dental hospital at the undergraduate training institute from August 2019 to February 2020. A total of 400 participants were provided survey questionnaires, who were 18 years and above. The patients included as participants, were randomly selected who were attending hospital for dental treatments in undergraduate course with 3rd, 4th and 5th year students. The consent form included a cover letter briefly describing the objectives and benefits of participation, however participation was voluntary. It was made clear that confidentiality will be maintained throughout the process.

The questionnaire consisted of 17 closed ended questions mainly divided in demographic data, distribution of patients based on their perception and belief towards dental treatment provided by dental students. The first section addressed the demographic information of participants; age range and category, employment status, education level, marital status and income category. The second section consisted of closed ended question related to undergraduate clinics method of booking, type of procedure received by the participant, method of contacting the student clinic by participants, reasons for taking dental student clinic appointment and previous experience with student clinics. The last section enquired about the feedback from the participants and their beliefs related to student clinics, including, satisfaction level and reasons for dissatisfaction, would they prefer repeating the experience, must students be supervised and are students able to manage emergencies.

The statistical analysis was performed using Statistical Package for Social Science Software (SPSS version 20) (IBM Corporation, New York, USA). Descriptive statistics (means, standard deviations, percentages, frequencies, tables) calculated to assess the student's awareness.

One-way analysis of variance (ANOVA) followed by post hoc Tukey test was performed for comparing different variables of study. Chi square test was employed to compare association between categorical variables.

RESULTS AND DISCUSSION

Demographic features: The total completed response were 268 out of 400 with a response rate of 67%. Table 1 shows that 67.2% of participants were males, while 32.8% were females. 42.9% were aged between 18-28 years, 23.1% were aged between 29-39 years, 20.5% were aged between 40-50 years, and 13.4% were 51 years and above. According to employment status, 30.2% of participants were students, 27.6% were governmental employee, 14.6% private sector, 13.1% were on-employed, 10.4% were retired, and 4.1% did freelancing. According to educational level, 38.1% had a bachelor's degree, 29.1% completed secondary, 16.8% had diploma, 7.1% with intermediate, 6% completed masters or higher, and 3% with primary or less .And their distribution according to marital status was, 43.3% were single, 52.6% were married, 3% were divorced and 1.1% were widowed. Finally, their distribution according to monthly income in Saudi riyals (SR) included, 32.1% of the participants' had monthly income of less than 3000, 21.3% were between 10000-19999, 20.1% were between 6000-9999, 17.2% were between 3000-5999, and 9.3% were 20000 and above.

Table 1. Distribution of the participants according to the demographic data.

		Frequency N= 268	Percent 100%	P-value
Gender	Male	180	67.2	.000
	Female	88	32.8	
Age	18-28 years old	115	42.9	.000
	29-39 years old	62	23.1	
	40-50 years old	55	20.5	
	51-60 years old and above	36	13.4	
Employment status	Student	81	30.2	.000
	Governmental employee	74	27.6	
	Private sector	39	14.6	
	Non-employed	35	13.1	
	Retired	28	10.4	
	Freelance	11	4.1	
Highest degree or level of school	Primary or less	8	3.0	.000
	intermediate	19	7.1	
	secondary	78	29.1	
	diploma	45	16.8	
	bachelor degree	102	38.1	
	masters or higher	16	6.0	
Marital status	Single	116	43.3	.000
	Married	141	52.6	
	Divorced	8	3.0	
	Widowed	3	1.1	
Monthly income	Less than 3000 SAR	86	32.1	.000
	3000-5999 SAR	46	17.2	
	6000-9999 SAR	54	20.1	
	10000-19999 SAR	57	21.3	
	20000 and above	25	9.3	

Chi-square test. P-value is significant at 0.01 level.

Patients perceptions towards treatments by dental students: Table 2 presents the distribution of participants according to the method of booking appointments with 48.1% through reception, 36.2% through the dental student and 15.7% by the waiting list. In addition, with regards to method of contacting patients, 64.2% of participants contacted treating dental student by text messages, 25.4% by phone calls, while 10.4% did not have any way of direct contact (Table 3). In addition, 82.1% of participants already had treatment experience

with undergraduate student, while 17.9% never had prior student treatment experience (Table 4).

Table 2. Presents participants' distribution according to the method of booking an appointments at the undergraduate clinics.

	Frequency	Percent	P-value
Reception	129	48.1	.000
Through the dental student	97	36.2	
Waiting list	42	15.7	
Total	268	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 3. Presentation of participant distribution according to the way they contact with the treating dental student.

	Frequency	Percent	P-value
Texts	172	64.2	.000
Phone calls	68	25.4	
I don't have any way of contact	28	10.4	
Total	268	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 4. Presentation of participant distribution, as to whether they have been treated by an undergraduate dental student previously.

	Frequency	Percent	P-value
Yes I have	220	82.1	.000
No it's my first time	48	17.9	
Total	268	100.0	

Chi-square test. P-value is significant at 0.01 level.

Regarding the reasons, why participants choose to undergo treatments by students, 45% believed that they would receive the optimal treatment under the supervision of specialists, 25.5% trusted the dental student as he or she was a relative or a friend, 17.3% for low or no cost of treatment, 10.5% were transferred by the specialist, and 1.8% got the idea form advertisement through social networking sites (Table 5). Participant's satisfaction levels from undergraduate treatments included, 59.5% evaluated the experience as very good, 30.5% evaluated the experience as good, 8.2% evaluated the experience as OK, and 1.8% evaluated the experience as poor (Table 6). Furthermore, out of the participants with not satisfactory treatment experience with students, 63.6% reported that the treatment took a long time and multiple appointments, 27.3% reported that the student lacks self-confidence and show anxiety, however 9.1% were dissatisfied with the final results of treatment (Table 7).

Table 5. Distribution of Reasons, why participants undergo treatment by the dental student.

	Frequency	Percent	P-value
The dental student was a relative or a friend of mine	56	25.5	.000
My believe that I will be provided by the optimum treatment under the supervision of specialists	99	45.0	
I was referred by a specialist	23	10.5	
Could not afford other solutions	38	17.3	
An advertisement on social media caught my attention	4	1.8	
Total	220	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 6. Presentation of participant satisfaction levels distribution on treatment by undergraduate dental student.

	Frequency	Percent	P-value
Very good	131	59.5	.000
Good	67	30.5	
Ok	18	8.2	
Poor	4	1.8	
Total	220	100.0	

Chi-square test. P-value is significant at 0.01 level.

The distribution of treatment procedure provided by the students included, caries removal and fillings (45%), root canal treatment (22.7%), calculus removal and teeth cleaning (12.3%) , fixed dentures (crowns and bridges) (8.6%), removable dentures (8.2%), and extraction or surgery (3.6%) (Table 8). Interesting, 90% of the participants preferred to repeat the experience with the same dental student, while 10% requested to change the treating clinician if possible (Table 9). A majority of participant believed that dental student should be supervised by the dental specialist throughout the treatment duration (60.9%), however, 39.1% of patients thought that supervision was not necessary (Table 10). Finally, 50.7% of patients considered that students are

capable of managing emergencies in the clinic, 40.7% responded as " I don't know" and 8.6% do suggested that students were not able to manage emergencies on their own (Table 11).

Table 7. Distribution of Reasons for dissatisfaction of participants with the treatment provided.

	Frequency	Percent	P-value
The anxiety and lack of confidence of the student.	6	27.3	.000
The treatment took a long time and multiple appointments.	14	63.6	
I didn't like the final	2	9.1	
results			
Total	22	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 8. Participant distribution based on type of dental treatment received.

	Frequency	Percent	P-value
Caries removal and fillings	98	44.5	.000
Root canal treatment	50	22.7	
Calculus removal and teeth cleaning	27	12.3	
Removable dentures	18	8.2	
Fixed dentures (crowns and bridges)	19	8.6	
Extraction or surgery	8	3.6	
Total	220	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 9. Participant distribution according to whether they prefer to repeat the experience with the same dental student.

	Frequency	Percent	P-value
Yes	198	90.0	.000
No	22	10.0	
Total	220	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 10. Distribution of participants according to their belief that the dental specialist should supervise the dental student for the time of treatment.

	Frequency	Percent	P-value
Yes	134	60.9	.001
No	86	39.1	
Total	220	100.0	

Chi-square test. P-value is significant at 0.01 level.

Table 11. Distribution of participants based on their belief that a dental student is capable of dealing with any emergency that could happen during the treatment.

	Frequency	Percent	P-value
Yes	136	50.7	.000
No	23	8.6	
I don't know	109	40.7	
Total	268	100.0	

Chi-square test. P-value is significant at 0.01 level.

This study provides information about the patients' perception towards receiving dental care from undergraduate students at the College of dentistry. One of the main aims of dental treatment is to provide comfort and function to satisfy patient. It is the most important element to measure the success of the delivered treatment (Mericon & Yon, 2002). Measurement of satisfaction is therefore as an integral aspect of evaluating the given care (Albiladi et al ., 2019). In recent years, quality of health care and its perception by patients has been an issue that gained some noticeable importance by Health Service Providers, therefore patient satisfaction has been considered as a good predictor of treatment compliance and adherence to care provider instructions. It is also considered as a tool for assessment in which patients' opinions could be used for service improvement and development.

In order to assess the quality of care provided by the students, participants were asked to evaluate their experience, in which the majority showed high levels of satisfaction with a percentage of 59.5% (very good). Thus indicating the high professionalism and management levels that students perform among their patients during clinical sessions and their ability to achieve their duties in a safe and ethical manner (Molina & Fernandez, 2017). Looking into the minority who were somewhat unsatisfied, the factor of long treatment duration was the main reason, showing the direct relationship between patient's own time and willingness to proceed with treatment.

This is previously proved in a study by Anderson et al., (Anderson et al., 2007). It is fair to claim that, the more the patients need to invest in their own resources (time)

the less valuable the outcome becomes, leading to less satisfaction35. In order to ensure a good flow of patients for dental students to practice on, it is crucial to take into consideration patients' personal perceptions towards health care in general and specifically oral health care. Our study highlighted the reasons that cause both patient adherence and reluctance to dental students. It is assumed that the selection of dental clinics is based on dental accessibility, convenience, and affordability.

In the present study the main reason of seeking treatment with students (45%) was due to supervision by specialists which shows to enhance the patient's trust in the student, as the clinical instructor closely supervises the dental student (Albiladi et al., 2019).Patients' loyalty is correlated with their compliance in taking the medical advice, use of medical services wisely and take a role in their medical care (Macstravic, 1995). The definition of patients' loyalty is the intention to visit the same doctor (Cyr, 2008). Among the 286 participants is this study, 90% preferred to repeat the experience with the same dental student, while 10% did not prefer to repeat it with the same dental student.

While clinical practice is considered as an integral part of educational process in dentistry, supervision plays a major role in enhancing student's learning to complete their academic programs successfully carrying with them good experience (Deuchar, 2008, Lee & Mckenzie, 2011). Clinical supervision by experts can markedly improve and build students clinical skills. On the other hand, it can obstruct it or inhibit it (Lingard et al., 2012). It was enquired from participants' if the student should be supervised by the dental specialist the whole time of the treatment, 60.9% believed that the dental student should be supervised by specialist the whole time of the treatment, while 39.1% answered that it is not necessary. Finally, dealing with emergencies becomes a challenging task (Gururaju et al., 2013).

One of the most commonly occurring medical emergencies is syncope (Gururaju et al., 2013). Therefore, Emergencies in dentistry include severe pain, abscesses and swelling (Dailey & Martin, 2001). Among our sample 50.7% of the participants believe that student is capable dealing with any emergency that could happen during the treatment, 8.6% do not believe, and 40.7% do not know. Although the present study suggests that patients have a positive attitude towards seeking treatment from undergraduate student clinics, however they still prefer supervised students. These findings should be considered in light of the possible limitations of the study. The findings reflect the opinion of the population involved and cannot be generalized to other populations. In addition, as a survey based study, it is difficult to remove all subjective bias of individuals involved.

CONCLUSION

Within the study limitations, two-thirds of the patients were satisfied with the dental treatments provided by students under supervision and the main reason for dissatisfaction was the delay and long duration of treatment. A majority agreed that they would seek treatment from dental students again and one-third of patients believed that students do not need supervision at all times. Further studies with larger sample size and different undergraduate institutes should be performed to validate these findings.

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Technological Qualities of Grain of Winter Crops Depending on the Sowing Time and Weather Conditions

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ABSTRACT

Grain quality is considered an important indicator of the level of development of grain production in any country. Thus, as a result of analyzing the technological indicators of grain quality, we can summarize that in different meteorological conditions, winter rye forms grains of class 3-4 quality, winter wheat and winter triticale - class 3. Dry development and ripening of the grain (HTC less than 1) ensures grain better in nature and vitreousness compared to over moistened years, but does not affect its classiness. The quality of the grain depends on the correct choice of the sowing time of winter crops. In the present study, the experiments were laid out according to generally accepted methods. To determine the technological qualities of grain, samples of the harvest of 2015 and 2016 were taken. Analyses were carried out in the testing laboratory of Perm Agro Service LLC.

KEY WORDS: WINTER RYE, WINTER WHEAT, WINTER TRITICALE, SOWING TIME, GRAIN QUALITY..

INTRODUCTION

Grain quality is considered an important indicator of the level of development of grain production in any country. There is a problem of insufficient production of bread flour with high technological qualities (Altukhov, 2005; Ismagilov, 2010; Chubenko, 2013). The results of studies by foreign and Russian scientists show that sowing time and weather conditions have a significant impact on crop yields and grain quality (Vasiukov et al., 2008; Kildiushkin et al., 2010; Peremecheva et al., 2007; Tikhonova & Fatykhov, 2013; Schönberger, 2000; Leszynska & Noworolnik, 2002, Pasynkovet al., 2017 Gwamba et al 2019).

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The quality of the grain depends on the correct choice of the sowing time of winter crops. So, when sowing winter wheat at an optimal time (the first decade of September) in Kemerovo, Volgograd, and Kursk regions, there is an increase in the protein and gluten content by 1–2.5% compared with earlier or late periods (Balashov & Malakhova, 2012; Egushova et al., 2012; Lazarev & Kotelnikov, 2015). In Smolensk, Nizhny Novgorod, and Irkutsk regions, the optimal period is the third decade of August (Ziuzina et al., 2013; Sultanov, 2014; Torikov & Ptitsyna, 2017; Shchennikova, and Kokina, 2018). Thus, the optimal sowing time for obtaining quality grain in each region is different, and its establishment is an important task.

MATERIAL AND METHODS

Field studies were carried out in 2014 - 2016 on the educational and experimental field of Perm State Agricultural Academy. The soil of the plot is sod-small podzolic heavy loam, moderately cultivated. Agrotechnical experience corresponds to the scientific system of agriculture recommended for the Cis-Urals (Akmanaev et al., 2012). The precursor is annual herbs

for green fodder. Objects of study - winter rye varieties Falonskaya 4, winter wheat Moskovskaya 39, and winter triticale Izhevskaya 2. The seeding rate of winter rye and wheat is 6 million viable seeds per hectare, winter triticale - 5 million viable seeds per hectare. Sowing was carried out in seven terms (Table 1). The experiments were laid out according to generally accepted methods (Dospechov, 2011). To determine the technological qualities of grain, samples of the harvest of 2015 and 2016 were taken. Analyzes were carried out in the testing laboratory of PermAgroService LLC. The technological qualities of grain were evaluated in accordance with GOSTs (GOST 34023-2016. Triticale. Technical conditions, 2017; GOST R 52554-2006. Wheat. Technical conditions, 2008; GOST R 53049-2008. Rye. Technical conditions, 2011; GOST R 54895-2012, 2013).

Table 1. Sowing time

sowing. term No	Planned sowing term	Actual sowing term	
		Second establishment 2014	third establishment 2015
1 (k)	August 15	August 15	August 14
2	August 18	August 18	August 21*
3	August 21	August 21	August 24*
4	August 24	August 24	August 29*
5	August 27	August 28*	September 4*
6	August 30	September 2*	September 10*
7	September 2	September 8*	September 12*

*changes in planned sowing time occurred due to heavy precipitation.

Meteorological conditions during the years of research differed in temperature and precipitation. Vegetation period 2014 - 2015 was characterized by moderately warm weather. In the autumn, the drop of average daily air temperature under +5° was observed in the first decade of October. In the winter and spring period of plant development thaw observed, which led to an intensive consumption of nutrients for respiration and created conditions for the plants to dry out. In the first half of the spring-summer period 2015, favorable temperature conditions developed for the development of winter crops, but the grain ripening period was characterized by a lowered background temperature and high humidity with a hydrothermal coefficient (HTC) of 1.98, which affected the quality of grain. Agroclimatic conditions 2015-2016 were favorable for the growth and development of winter crops. The end of the autumn growing season also was in the first decade of October. The winter was relatively warm, and the snow melted early. The spring-summer period was characterized as warm and dry. Since the third decade of April, there was a lack of precipitation, the HTC during the period of maturation was 1.1. This ensured the earlier ripening of winter crops, favorable conditions for harvesting and the formation of higher quality grains.

Table 2. Technological quality of winter rye grain

Sowing term	Nature, g/l		Falling number, s.	
	2015	2016	2015	2016
1 (k)	639	719	61	89
2	647	728	61	84
3	654	722	61	81
4	655	715	61	83
5	656	706	61	77
6	641	686	61	68
7	635	685	61	68
Mean	647	709	61	79

RESULT AND DISCUSSION

In 2015, under unfavorable conditions for grain ripening, the nature of winter rye averaged 647 g/l, which corresponds to the third class of GOST (Table 2). In terms of sowing, the quality varied from the third class, when sowing from the second to the sixth term (641 - 655 g/l), to the fourth class - in the first and seventh terms of sowing (635 - 639 g/l). In terms of the number of fall grain winter rye corresponded only to the fourth class (61 s.). In 2016, under favorable conditions during ripening, the nature of winter rye grain averaged 709 g/l (first class of GOST). According to this indicator, when sown from the first to the fifth term, the grain also corresponded to the first class of GOST for winter rye grain (not less than 700 g/l), while the sixth and seventh term - to the second class (not less than 680 g/l). In terms of the number of falling when sown from the first to the fourth term, the grain corresponds to the third quality class (81 - 89 s.). In other terms - to the fourth class. Thus, the quality of winter rye grain in the Middle Urals is limited by the influence of weather conditions. In terms of the number of falling, it corresponds to the fourth grade. Grain of the third class can be obtained only under favorable weather conditions and sowing time from August 14 to 29.

The quality of winter wheat grain also depends on weather conditions (Table 3). In 2015 it was lower in terms of the nature of the grain, on average, corresponded to the third class of GOST (744 g/l). When sown in the fifth and sixth terms, the grain corresponded to the first class, and in the remaining periods - to the third class. The conditions of 2016 allowed for a larger grain of winter wheat to form. The nature of the terms varied within 794 - 802 g/l, which corresponds to the first class of GOST. In terms of the number of falling, grain of winter wheat in 2015 and 2016 corresponded to the requirements of the first class of GOST, regardless of the sowing period. Grain vitreousness met the requirements of the third class in 2015 and the first class in 2016 when sown from the

first to the sixth term, and to the third class with the seventh term of sowing.

An important parameter of wheat grain quality is the gluten content. The mass fraction of raw gluten varies depending on weather conditions. The highest percentage on average was observed in 2016 - 31%. In 2015, it was 29.6%. The gluten content, depending on the sowing date, varies in different ways and corresponds to 1 - 3 classes of GOST. The qualitative assessment of gluten indicates that all the samples for the studied sowing dates over the years of research corresponded to the second group of quality - satisfactorily weak. The readings of the FDM device were 76-85 units. Thus, the technological quality of grain, regardless of weather conditions and sowing time, is limited by the quality of raw gluten and corresponds to the third class of GOST. According to other indicators in favorable years, the grain of winter wheat meets the requirements of the first class. In 2015, the nature of winter triticale grain for all sowing terms was 556 - 593 g/l, and corresponded to the third class of GOST (Table 4). In 2016, triticale grain was formed under dry conditions, the grain nature obtained was of first class when sown from the second to the fifth term (702 - 713 g/l). In the first and sixth terms, the grain corresponded to the second class, and in the seventh term of sowing - to the third class.

Table 3. Technological quality of winter wheat grain

Sowing term	Nature, g/l		Vitreousness, %		Falling number, s.		Mass fraction of raw gluten, %		FDM. index, un	
	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
1 (k)	735	802	53	65	401	318	33.0	27.0	76	76
2	737	802	55	69	273	299	29.0	27.0	90	80
3	739	801	52	66	260	306	32.0	33.0	80	85
4	748	800	53	72	324	345	26.0	29.0	76	76
5	764	794	56	66	331	351	26.0	32.0	85	85
6	751	794	53	64	311	326	31.0	34.0	85	90
7	736	781	55	58	256	341	30.0	35.0	80	90
Mean	744	796	54	66	308	327	29.6	31.0	81	83

Table 4. Technological quality of winter triticale grain

Sowing term	Nature, g/l		Vitreousness, %		Falling number, s.	
	2015	2016	2015	2016	2015	2016
1 (k)	-	697	-	52	-	68
2	593	709	43	58	61	66
3	592	712	50	65	61	59
4	556	713	53	68	61	66
5	582	702	48	63	61	68
6	566	690	48	65	61	67
7	558	661	50	63	61	63
Mean	492	698	47	62	61	65

Regardless of the year and time of sowing, the vitreousness of winter triticale grain corresponds to the first class of GOST. In 2015, this figure ranged from 43 to 53%; in 2016, the vitreousness of grain was high (52-68%). In terms of the number of falling, the winter triticale grain corresponds to the third class, regardless of weather conditions and sowing time. In 2015 it averaged 61 s., in 2016 - 65 s. Thus, it is possible to obtain third-class triticale grain.

CONCLUSION

Thus, as a result of analyzing the technological indicators of grain quality, we can summarize that in different meteorological conditions, winter rye forms grains of class 3-4 quality, winter wheat and winter triticale - class 3. Dry development and ripening of the grain (HTC less than 1) ensures grain better in nature and vitreousness compared to overmoistened years, but does not affect its classiness.

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Breast Cancer Detection: Analysis by Wideband Antennas

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ABSTRACT

Calculation of the numerical values of the depth of cancer tissues have been presented in the cancerous organs. In this work we have developed a wideband antenna to detect the cancerous organs. An EF shaped wideband antenna with 2x2 grid pattern has been designed with its geographical coordinates by mathematical modelling; simulated by Advanced Design Simulator (ADS) using Moments of Methods (MoM); fabricated their design in the form of a prototype using Microwave Monolithic Integrated Circuits (MMIC); and analysed by Agilent N99917A Microwave Analyser, which has been represented in this primary goal of research. It comprises a compact dimensional of 18.64x17.92x1 mm3. It includes the 2x2 array pattern with four EF shaped radiating slots, which printed on the 1-millimetre dielectric thickness of FR4 material and the conductive ground plane. It has been delivered at 5.417 GHz operational frequency, which covers the wideband spectrum of 4.89 GHz to 6 GHz. Furthermore, it has a wideband antenna, a low-profile structure with four numbers of radiating slots. These wideband antennas have been implemented for breast cancer detection application, which is represented by the secondary delivery of research. These wideband antenna with human breast equivalent dielectric model under existing Debye testbeds. These wideband antennas have impressed on the coupling materials, which act as the coupling medium. They have provided better scattering results than air medium. These coupling materials have presented higher and lower dielectric strengths respectively. The average of scattered responses has gathered on it. It has determined the depth of cancerous tissues presented in the cancerous organ with the help of velocity of propagation, the distance between the cancerous organ and transceiver antenna, dielectric strength and travelling time of scattered responses respectively. Therefore, these scattered responses have been obtained from the cancerous organs under Debye testbed. Hence, the numerical value of depth and spreading the coverage of depth in cancerous organ is validated under existing Debye testbeds.

KEY WORDS: BREAST CANCER, 2X2 ARRAY PATTERN; EF SHAPED PROTOTYPES; MICROSTRIP PATCH ANTENNA.

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INTRODUCTION

The microwave spectrum has been used during past twenty decades in medical, industrial and scientific applications. The microwave imaging is one of the most suitable breast cancer detection techniques. Its main objective is the hypothesis that the dielectric properties, electrical properties, impedance matching, conductivity, permittivity of the cancerous breast's tumor tissues are

slightly different from the normal breast tissues and the cancerous organ's tissues. Microwave frequencies are millimetre wavelengths of the electromagnetic spectrum. The MI system provided the non-ionizing and non-invasive approaches and showed reasonable penetration in the breast tissue surrounding of breast organ. The microwave imaging techniques were used in one pair of antennas (transmitter, receiver antenna).

The transmitter transmitted the microwave signals to the breast and the internal tissues surrounding it. The receiver side scattered signals were reflected on it, which were gathered by the receiver antenna. The cancerous (malignant tumors) and normal breast tissues have different dielectric permittivity and conductivity properties. Microwave signal is reflected by the cancerous tissue and the scatter signal will collect from the body as differently. Microwave signals through diagnosis trials detected a malignant tumour of 5-10 millimetre range with an accuracy of 85%. Moreover, the ultra-wideband microwave imaging systems have been used to examine the breast cancer diagnosis, (Meaney et al., 2000, Fear et al., 2002, Gibbins et al., 2009, Sakthisudhan et al., 2020).

A wideband antenna has been operated under 4.5 GHz-10 GHz microwave frequencies. It was designed by cavity –backed patch antenna with a 3D array pattern which was replaced by monopole antennas with a 2D array pattern. The microwave imaging system, focusing on the following challenges and issues were discussed as, i)Ultra-Wide-Band antennas, ii) Compact size, iii) Steerable antennas, iv) Interference rejection factors,and v) Improved radiation efficiency. Chu Yu et al (Yu et al., 2008) examined a prototype model for Microwave Imaging systems. This system consisted of one pair of dipole antennas to avoid mutual coupling losses between two ports.

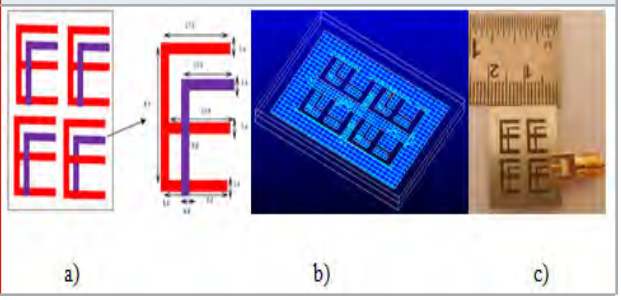
This proposed system can effectively detect the dielectric size of object is 5 mm at 1.74 GHz imaging frequency. Similarly, the various microstrip patch antennas of surveys have demonstrated the various test beds such as, Dipole Antennas; Dielectric Resonator Antennas; Patch antennas; Slot antennas; Vivaldi antennas; Horn antennas and MEMS Micro electro mechanical systems -steerable antennas (Woten and El-Shenawee, 2008, Shi et al., 2009, Amineh et al., 2009, Huang and Kishk, 2009, Gibbins et al., 2009, Al-Joumayly et al., 2010, Bourqui et al., 2010, Hutchings and El-Shenawee, 2010, Amineh et al., 2010, See et al., 2012, Sakthisudhan et al., 2020).

The Microwave Imaging system, coupling materials are mandatory to reduce the mismatching between the antenna systems and the breast tissue. Transverse. The comparative study of slot antennas and stacked antennas were used in the imaging systems (Sakthisudhan et al., 2020).In this research the microstrip patch antenna design and fabrication have been found suitable for cancerous diagnosis applications.

MATERIAL AND METHODS

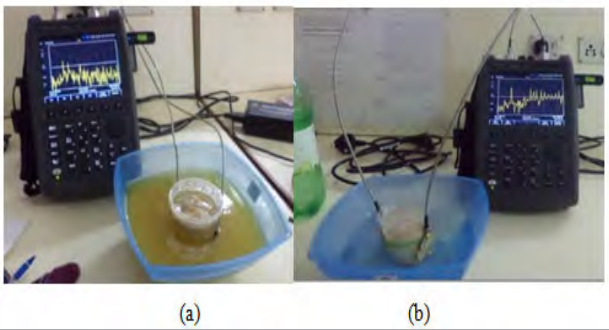
A 2x2 array pattern of EF shaped Microstrip patch antenna has been illustrated in Figure 1. The proposed prototype is to be required the following design parameters, such as, selection of 5 GHz resonator frequency; FR4 dielectric substrate with dielectric constant is 4.6; and thickness of dielectric layer is 1mm. It consists of conductive strip of 12.64x17.9 mm2with mounted on 1 mm dielectric layer thickness. Therefore, it achieves a compact design of 18.64x24.9x1 mm3dimensional area.

Figure 1: a) geometric coordinates (units in milimeter); b) simulation snapshot & c) fabricated prototypes of EF Shaped with 2x2 grid pattern.



A proposed design is designed by mathematical modelling equation. The excitation port is etched on the ([a] matrix element) a23EF shaped element of the array pattern via 50Ω of RF-SMA connectors. The proposed structure is fabricated with 4.6 dielectric constant with 1 mm thickness of FR4 material, mounted on the conductive ground plane strip via Surface Mount Adaptor (SMA) connector. The SMA connector is measured by Microwave Analyzer (Agilent N99917A) and this testing results are reliable and compared with the simulated prototype.(Sakthisudhan et al 2016, 2020).

Figure 2: Proposed prototypes impressed in coupling materials a) higher & b) conductive medium



A delivery of secondary research, the proposed prototypes with dielectric phantom model has been examined the scattered signal, which is illustrated in Figure 2. The dielectric phantom model consists of human equivalent model. It consolidated the different biological contents, which dielectric strengths are plotted in Table 1. Hence,

these test bed have delivered the four different stages of results, they are early stage; minor stage, growing stage and major stages respectively.

Table 1. Contents of breast phantom model

S.No	Dielectric Materials	Biological Breast Contents	Standard dielectric strength	Equivalent dielectric strength	Dielectric strength examined by measurements
1.	Human Breast Model	Cancerous Tissue	42-45	Coal Piece	43
2.		Breast Tissue	27-35	Wheat Flour	27.654
3.		Skin Layer	36-42	Glycerin	35.129
4.		Blood Components	50-60	Big Sugar Sugar	53.2 51.5
5.	Textile Woven Materials	Cotton Woven	3.9-7.5	Cotton	4.83
6.		Polyester Woven	2.8-4.5	Polyester	3.8
7.	Coupling Materials	Glycerin	43	Glycerin	40.41
8.		Vegetable Palm Oils	3.75	Vegetable Palm Oils	3.87

Table 2. Performance study of proposed 2x2 Array of EF Microstrip slots and Existing slots

		Existing Microstrip slots			2x2 Array of EF Microstrip slots			
S. No	Antenna Parameters	Simulation design	Fabricated prototype	(Denidni et al., 2008)	(Hazra et al., 2013)	(Liu et al., 2010)	(Liu et al., 2011)	(Archevapanich et al., 2007)
1.	Shape of Patch Strips	2x2 Array of EF Shaped	E Shaped	P Shaped	Inverted L Shaped	Inverted L Shaped	E Shaped	
2.	Resonance f_r (GHz)	5.156	5.417	5.8	2.45	2.42, 5.2	2.3, 3.6, 5.04	2.46, 5.3
3.	Return Loss S_{11} (dB)	17.473	21.274	<10	17.5	<10	>20	40.31
4.	Reflection Coefficient ()	0.13	0.09	0.316	0.133	0.316	0.1	0.1
5.	VSWR Ratio	1.31	1.19	1.92	1.3	1.92	1.22	1.22
6.	Reflected Power (%)	1.69	0.81	9.9	1.8	9.9	1	1
7.	Reflected Power (dB)	-17.7	-20.9	-10.03	-17.45	-10.03	-20.08	-20.08
8.	Mismatch Loss (dB)	0.08	0.03	0.45	0.08	0.45	0.04	0.04
9.	Non Reflected Power- dB	0.9665	0.9838	0.9	0.98	0.9	0.99	0.99
10.	Bandwidth Coverage GHz	4.8 to 6	4.89 to 6	4.8-6	2.35-2.25	2.34-2.55 4.8-7.2	2.14-2.52 2.82-3.74 5.15-6.02	2.4-2.52 4.82-6.32
11.	Fractional BW (%)	23.27%	20.49%	20.69	4.08	8.67,46.1	16.5, 25.56, 17.3	4.87 28.3
12.	Antenna Q Factor	4.3	10.8	4.83	24.5	97.1	5.79	3.53
13.	Dimension of Design (mm^3)		18.7x23.9x1 x5.78	15x25 x1.52	110x158.5	25x30x05	20x30	27x17.3x 1.575

RESULTS AND DISCUSSION

The comparison of the fabricated MPA prototype with the simulated MPA design is shown in Figure 3. The return loss of 17.473 dB has been achieved at a resonant of 5.156 GHz and coverage of wide band of 4.8 to 6 GHz in the simulation structure. The return loss of 21.274 dB has been obtained at a resonance of 5.417 GHz, coverage of 4.89 to 5.3 GHz in the fabricated MPA prototypes. A comparison of the proposed MPA with the existing MPAs is listed in Table 2. MPAs require the UWB band of frequencies and ISM application standards. The fabricated MPAs results are justified with that of the simulated MPAs design and has improved FBW than the simulated MPA design. Hence, it is called as wideband prototypes (Sakthisudhan et al 2020).

Figure 3: Comparison of fabricated & simulated prototypes

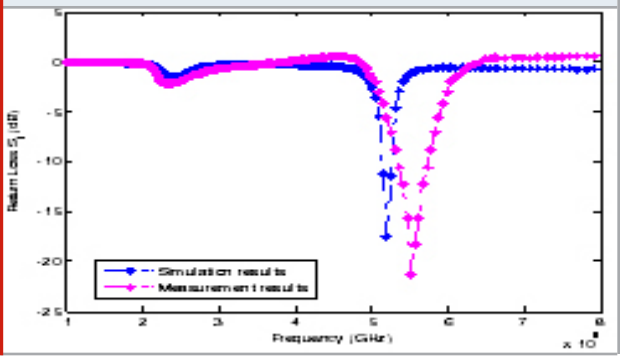
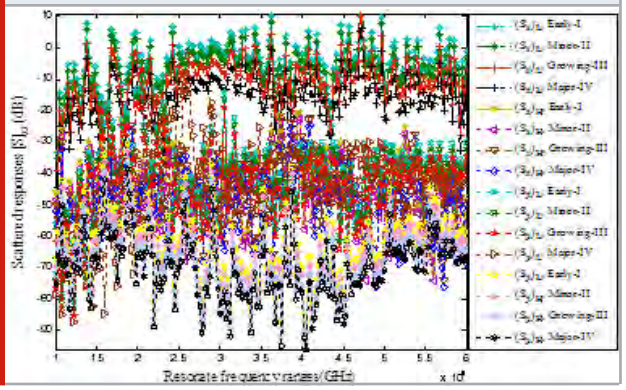


Table 3. Analysis of tumor characteristics of microstrip slot of EF with an Existing Debye Test Beds

	Category of Screening stages	Depth of tumor At Major Stage Frequency f_{major} (GHz)	Depth of tumor At Early Stage Frequency f_{early} (GHz)	Travelling Time δt (ns)	Proposed microstrip antennas			(Fear et al., 2002) Analyzed in Existing Debye Test Beds Parameters			Physical Distance Between the Tumor with MPAs (cm)
					Dielectric Strength of Tumor	Velocity of Propagation (mm/s)	Depth of Tumor by Numerical Analysis (mm)	Dielectric Strength of Tumbeen or	Velocity of Propagation (mm/s)	Depth of Tumor by Numerical Analysis (mm)	
Proposed MPAs Based Debye Test Beds	Early-I 1cm	5	3.4	0.625	48.5	$4.3x10^{10}$	53.75	9	10^{11}	125	129.8
	Minor-II 2cm	5	3.45	0.65	48.5	$4.3x10^{10}$	55.9	9	10^{11}	130	127.4
	Growing-III 2.5 cm	5	3.5	0.667	48.5	$4.3x10^{10}$	57.36	9	10^{11}	133.4	109.2
	Major-IV 3 cm	4.99	3	0.503	48.5	$4.3x10^{10}$	43.26	9	10^{11}	100.6	110.4
(Kiruthika and Sharma, 2011)	5	3.4	0.625	21.3	$6.5x10^{10}$	81.25	9	10^{11}	125	129.8	129.8
	5	3	0.503	21.3	$6.5x10^{10}$	65.39	9	10^{11}	100.6	127.4	127.4
	5	3.45	0.65	21.3	$6.5x10^{10}$	84.5	9	10^{11}	130	110.4	110.4
(Salvador and Vecchi, 2009)	5	3.4	0.625	20 to 40	$5.3x10^{10}$	66.25	9	10^{11}	125	129.4	129.4
	4.99	3	0.503	60 to 80	$3.1x10^{10}$	31.186	9	10^{11}	100.6	109.2	109.2

Figure 4: Scattered Signals have gathered by Existing Debye Test-beds



These proposed microstrip slots have been evaluated by the Existing Debye test bed setup, illustrated in Figure 2 (a & b).It consists the pair of transreceiver antenna with human's dielectric equivalent breast model. These scattered responses have examined under lower and higher coupling medium respectvely. Since, these proposed slots have impressed in these coupling medium. Figure 4 has gathered scattered signals from different resolution stages under Debye test beds. Finally, these results have analysed and segregated with dielectric equivalent of healthy organs. These results have described the early, minor, growing and major stages of cancerous organ respectively. Therefore, the spreading cancer tissues, depth has calculated in the Table 4.

CONCLUSION

A 2x2 grid pattern with EF shaped fabricated prototypes has been demonstrated and validated by Network Analyzer in this research. Moreover, these prototypes have been compared with the existing prototypes. It has delivered the resonant frequency of 5.417 GHz, wide band spectrum of bandwidth 4.89 to 6 GHz. The different tumor resolution characteristics have been demonstrated under the existing Debye model. Hence, these data have provided the clinical diagnosis of the breast cancer. Furthermore, these analyses have offered the depth of

cancerous organ under different resolution stages, which can be used suitably by radiology professionals.

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A Suggested Model to Raise Awareness of Cybersecurity Among Computer Teachers in Public Education: An Analytical Study on Education Department in Jeddah Governorate

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ABSTRACT

The study aimed at presenting a proposed model to raise awareness of cybersecurity among computer teachers in public education based on a set of previous studies, the most important of which was carried out by the researcher. The descriptive analytical approach was used to achieve the goal of the study, based on the nature of the study and its questions. The study concluded in its results that enhancing cyber awareness is based on educational training through identifying training needs related to cybersecurity and building and implementing training programs. In addition, the continuous training of the staff on duty is fruitful in raising awareness of cybersecurity hand in hand with holding effective partnerships in the field of information culture and information and cyber security. The study also identified a set of axes that contribute to raising awareness of cybersecurity through an analysis of existing and potential risks as well as indicating intended and unintended information crimes and measures that can be executed about them, and how to prevent and manage crises related to cybersecurity. Moreover, the study presented a set of ways and procedures through which to preserve cybersecurity through a set of basic security elements and means of awareness and digital culture, and finally establishing measures to build policies and legislation that guarantee the preservation of cyber security. The study recommends building educational specializations to teach information and cyber security, building curricula related to information and cyber security, and a continuous updating of the expected risks that may occur on cybersecurity and the development of plans facing them and crisis management and prevention plans. The study presented a suggested model based on its results and through reviewing previous studies aimed at raising awareness of cybersecurity among computer teachers in public education.

KEY WORDS: CYBER SECURITY, PUBLIC EDUCATION, COMPUTER, INFORMATION SECURITY, A SUGGESTED MODEL.

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INTRODUCTION

Cyber security is among the main challenges that has emerged with the accelerating development and progress of technology. By definition, it means that we must protect the conduct of cyber operations, protect data and applications, and preserve and maintain national information for individuals and the state. That is, to prevent any unauthorized entry or any tampering with data. Therefore, it is necessary to build a strong system that

protects these services and information that this beautiful technology provide us with through the cyberspace. As well, we should work on teachers' awareness, especially computer teachers. Indeed, cybersecurity has become the talk of the whole world, and has even become a political part of any other security, economic or political policies, as decision makers in various countries have put cybersecurity issues as a priority in their policy (Al-Otaibi, 2017).

As the cybersecurity becomes a subject of increasing public interest and research efforts, more and more studies that examine the roles of male and female teachers in particular have been conducted. These studies treat different subjects such as setting rules in the curriculum and classes to understand cybersecurity clearly, empowering students with problem-solving skills, providing peer counseling, and educating parents about cyber security through parent meetings and newsletters. However, cybersecurity is a very recent research topic in the Arab world that has not received enough attention at the level of scientific research. Nevertheless, it is not possible to neglect some of the few efforts, especially in the educational field and among teachers since it is one of the most important competencies for computer teachers. So, the current study is interested in finding a model to raise awareness of cyber security among secondary stage computer teachers in Jeddah.

The study issue: Through the experience of the researcher in the field of computers and based on his knowledge of previous studies, and based on his previous published study (Alsahafi, 2019) about the level of cybersecurity awareness among computer teachers in the education department of Jeddah and its results and recommendations according to the application of its curriculum and answering its questions and checking the validity of its hypotheses, the researcher decided to take advantage of these previous data to build a proposed model aiming at raising awareness of cybersecurity among computer teachers in public education by analyzing the results of his previous studies and inferring from previous studies and literature. The study seeks to answer the following question: "What is the suggested model for raising awareness of cybersecurity among female computer teachers in the Education Department in Jeddah Governorate?"

Previous Studies: Alsahafi's study aimed at identifying the level of cybersecurity awareness among secondary stage computer teachers in the city of Jeddah. The study community consisted of all computer teachers of the secondary stage in Jeddah for the academic year 1440 AH-2019 CE and their number was (352) teachers according to Jeddah Education Department statistics. The study sample consisted of (106) Female teachers. The quantitative method was used according to the study issue, questions, and nature. A questionnaire was used as a data collection tool. The study found in its results that there are weaknesses and limitations among computer teachers both in awareness of concepts of cyber security and in awareness of the level of cybersecurity. In addition, there is an absence of statistically significant differences between average responses of study sample individuals at the level of significance (≥ 0, 05) in the degree of cyber security awareness of computer teachers. This was due to the current study variables (years of experience - educational qualifications - training courses). The study recommended mainly:

1. The provision of free in-depth training programs in cybersecurity for computer teachers on top of work.
2. Attaching female teachers to diplomas in cybersecurity to raise their level of understanding, awareness and application.
3. The integration of cyber security in local educational programs.
4. Issuing and distributing circulars related to regulations and legislations developed for cybersecurity to all schools.
5. Integrating regulations and legislations within the code of career behavior.
6. Paying more institutional attention to educational seminars and training workshops that highlight the importance of developing cybersecurity.
7. The use of the Ministry of Education experts to develop cybersecurity tools in various educational institutions.

The study of Al-Ghadian et al., (2018) also aimed at revealing the most important forms of electronic blackmail crimes, their motives and their psychological effects from the viewpoint of teachers, Authority members and psychological counselors. The sample of the study consisted of (523) members divided into three groups: The first comprised the (48) members of the authority for the Promotion of Virtue and Prevention of Vice. The second consisted of (48) psychological counselors. The third was formed by (368) randomly chosen male and female teachers. To achieve the study objectives the researchers used three self-prepared criteria including the criterion of electronic crime images, the criterion of electronic crime motives, and the criterion of psychological effects of electronic blackmail after checking their psychometric characteristics.

The results indicated that there are statistically significant differences in the physical, emotional, and entertainment motives among the psychological counselors and the teachers in favor of the teachers, and between the teachers and the authority members in favor of the teachers. As for the differences in sexual motives, they were between the teachers and the authority members in favor of the authority members. Finally, the results of the study showed that there are statistically significant differences

in the degree of estimating the psychological effects of crimes of electronic blackmail due to the difference in the respondent category (male and female teachers and psychological counselors), and the differences were between psychological counselors and teachers in favor of psychological counselors.

Abdel Majid (2018) studied Cyber security is an urgent necessity for community security: The Safe Family Proposal for Educating the Arab Gulf Society in Information Security for Both Students and Parents aimed at revealing the role of parents in protecting their children from the threat of hacking and electronic blackmail. This study made a comparison between many families, parents and children, who had the opportunity to be properly qualified to use these technologies, and those families who were not trained to do so. This comparison was in order to identify the role of parents in protecting their children from electronic hacks.

The study found that there is a very important role for parents in protecting their children from electronic threats, including cases where children have an adequate level of education in dealing with this new technology. Therefore, the researcher has put forward a qualifying proposal for preserving the electronic privacy of male and female students, which includes all categories of public and private education, in addition to a program for qualifying both parents. This program seeks to contribute to the raising of the society security culture and the protection of children against this urgent danger.

Al-Shammari (2015) carried out a study on strategic vision to protect the cyberspace of the Kingdom of Saudi Arabia which aimed at: a) Clarifying the concept of cyberspace, its limits and its characteristics. b) Defining the electronic gap. c) Reviewing the reality of cyberspace in the Kingdom of Saudi Arabia. d) Explaining the dangers of cyberspace. e) Clarifying the awareness of those responsible for information security. f) Clarifying the obstacles that hinder the Kingdom's ability from confronting the dangers of cyberspace. The researcher used the descriptive analytical approach and Content analysis in addition to the deductive inductive approach. The study found that there are some risks that threaten the cyberspace of the Kingdom of Saudi Arabia. In addition, there are limits of the awareness of those responsible for information security in the Kingdom of these risks. It also found that there are many obstacles hindering the Kingdom's ability to confront those risks.

Recently, Nakama and Paullet (2018) aimed at teaching students at all university academic levels how to face cyber-attacks. The study indicated that there is another important set of key skills in the dynamics of online courses in the first university stages to overcome their contextual limitations: (1) Learn how to navigate the learning management system. (2) Send and receive messages effectively between students and faculty members. The study contributed to the students 'acquisition of online learning strategies because students may feel that they are unable to complete academic assignments without assistance, which can threaten their self-value. As a result, many college students fail to seek the needed help, considering it as embarrassing, accepting defeat, and something that can be avoided whenever possible. Through the success of the program, the study contributed to develop cybersecurity among university students among all academic levels.

Goran (2017) studied "Cyber Security Risks in Public secondary" which was aimed at analyzing cybersecurity problems in a public secondary school and suggest practical solutions. A sample of secondary schools was used through the case study methodology. The result of this paper was a case study on an urban secondary school and its weaknesses in front of various electronic attacks through clarifying the dangers of electronic attacks and how to prevent them.

METHODOLOGY

Based on the nature of the study, its issue, and its goals, the analytical method was used to analyze the data of previous studies and the study previously conducted by the researcher on the topic of cybersecurity awareness among computer teachers in the Education Department in Jeddah Governorate. It also used the focus groups method in order to amend and validate the proposed model presented by the study through a group of seven experts in the field of computer, information and cyber security.

RESULTS AND DISCUSSION

Through his previous study (Alsahafi, 2019) reached a set of statistical results that need to be enhanced in the proposed model because of their low application level through the previous study method:

First: Computer teachers' awareness of the concept of cyber security in Jeddah.

Table 1. The level of awareness of the Cyber security among computer teachers in Jeddah education department

Paragraph No	Paragraph Text	SMA	Standard Deviation	Response level
1	I know the risks of opening links and email attachments.	2.53	1.21	Medium
2	I have a solid knowledge of the concept of social engineering.	2.48	1.35	Medium
3	I know the risks of smart phone viruses.	2.34	1.14	Medium
4	I have an understanding of the concept of phishing (fraud) online.	2.21	1.08	Medium

Other phrases like: (I have full knowledge of the concept of social engineering - I have knowledge of the risks of smart phone viruses - I have knowledge of the concept of phishing (electronic fraud) got lower degrees in their average due to the lack of coverage of those concepts at the school community and by public media in contrast to other common concepts and terms that teachers know, although, without a precise definition of their dimensions.

Second: Computer teachers' awareness of ways to preserve cyber security systems in the city of Jeddah:

Table 2. Computer teachers' awareness of ways to preserve cyber security systems in the city of Jeddah

Paragraph No	Paragraph Text	SMA	Standard Deviation	Response level
1	I am aware of the features needed to create a good password when entering websites.	2.34	1.14	Medium
2	I use the same password for all social media and email.	2.18	1.48	Medium
3	Use the same password for the websites I need to conduct financial operations like bank websites and online shopping websites.	2.20	1.35	Medium
4	I know the danger of sending a password via e-mail.	2.11	1.44	Medium
5	I change the password regularly.	2.02	1.39	Medium
6	I read user agreements of free software before pressing "I agree".	1.95	1.14	Low
7	There is a separate law for cybercrime in Saudi Arabia.	1.87	1.48	Low
8	There are laws and regulations for cyber security in Saudi Arabia.	1.74	1.35	Low

The phrases (I read user agreements for a free program before pressing "I agree" - there is a separate law for cyber crime in Saudi Arabia - there are laws and regulations for cyber security in Saudi Arabia) got a low degree. This may be due to the fact that computer teachers perform many procedures and processes related to dealing with Software and computer applications, which leads them not to read the agreements and conditions of each program that they download and install on their devices. In addition, there was a lack of knowledge of the classifications of laws and legislations related to cybercrime. The result was expected, as it is common in societies and not limited to computer teachers who should be keen to understand the terms of the agreements.

3- A suggested model for raising awareness of cybersecurity among computer teachers in public education:

Preface: Based on the application of the study methodology, the results and recommendations that were presented, and through an analysis of the results of the previous study related to the topic that the researcher previously performed, and what was seen from previous studies that discussed the study topic, the researcher reached the following model, which was reviewed by experts who were chosen to discuss and amend it so as to reach its final wording as follows:

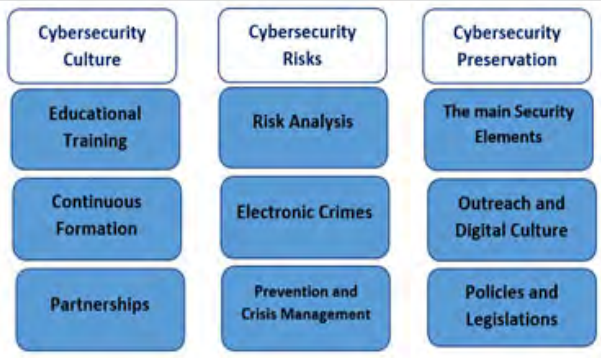
Vision: Reaching a public awareness that helps in achieving and enhancing cybersecurity, and clarifying some practical solutions through which information and cyberspace can be protected in relation to the growing threats and risks facing this vital field.

The objectives of the proposed model: The goals of the proposed model are:

1. Raising awareness of cyber security among computer teachers
2. Raising awareness of the importance of cybersecurity
3. Cybersecurity continuous training and formation
4. Identify partnerships related to raising awareness of cybersecurity
5. Factors affecting cyber security
6. The dangers of poor cybersecurity
7. Methods of preserving cyber security

The suggested model for raising awareness of cybersecurity for computer teachers in public education:

Figure 1



The first chapter: Cybersecurity culture

Educational training: It includes the following procedures: Analysis of training needs in the field of cybersecurity Determine minimum skills in cybersecurity Building training programs related to cybersecurity

2. Continuous Formation:

It includes the following procedures: Identify appropriate educational and specialized needs for cybersecurity Preparing internal and external scholarship plans and programs Identify continuous formation specializations related to cybersecurity Building systems and procedures that regulate continuous formation in a way that motivates female teachers Prepare training plans, select trainers, and schedule programs

3. Partnerships:
It includes the following procedures: Identify areas for partnerships in spreading a cybersecurity culture Identify cybersecurity relevant agencies and partners Build educational and training partnerships according to existing needs Build cooperation with relevant expertise agencies

The second chapter: cybersecurity risks:1. Risk analysis:
It includes the following procedures: Identify constant ongoing cybersecurity problems, and develop, remedy and update prevention plans Identify intended and unintended threats related to the internal and external misuse Identify and update global threats to cybersecurity Continuous perusal of security gaps revealed from time to time and keeping vigilant to dealing with them on time

2. Electronic crimes:
It includes the following procedures: Identify electronic crimes related to cybersecurity according to the legislation within the educational system or according to the system of electronic crimes related to cyber security Define procedures for dealing with electronic crimes according to their levels, whether dealing with them internally or externally as crimes that need the intervention of the competent authorities Issue sanctions against electronic crimes related to cybersecurity and circulate them to employees and affiliates Continuous monitoring, technically and administratively, of everything that poses a threat with regard to electronic crimes.

3. Prevention and crisis management:
It includes the following procedures: Spreading digital culture and raising awareness regarding the risks related to cybersecurity periodically for employees and affiliates Continuous updating of ways to prevent cyber security threats through casual situations or others' experiences Preparing predictive studies related to cybersecurity threats in the areas of administration, technology, legislation, laws and policies Preparing crisis response and management plans as an anticipation for every emergency in the field of cyber security threats.

The third chapter: Cyber security Preservation: 1. The main security elements:
The main elements include: Confidentiality and security: it means ensuring that the information is neither disclosed nor viewed by persons who are not authorized to do so.

Integrity and content safety: It is to ensure that the information content is correct and has not been modified, and in particular, it has not been destroyed, altered, or tampered with at any stage of processing or exchange, whether in the stage of internal dealing with the information or by unlawful interference. Constant availability of information or service: It must ensure that the information system continues to operate and the ability to interact with information and provide service to informational sites, and that the user will not be subjected to use or access prevention to the system. Do not deny the information-related behavior of the operator: It is intended to ensure that the person connected to the information or its sites cannot deny that he has acted, so that the ability to prove this behavior is available and that a person at a certain time has performed it, as well as the inability of the recipient of a specific message to deny receiving this message.

2. Outreach and digital culture: Digital citizenship: It includes The following units:

Respect which includes the following three criteria: Digital Access: It means working towards providing equal digital rights and supporting electronic access. Digital Etiquette: standards for behavior and procedures through the use of technology Digital Law: It means digital responsibility for actions and deedsEducation:It includes three criteria as follows:

Digital Communication: Everyone now has the opportunity to communicate and collaborate with anyone else in any part of the world at any time.

Digital Literacy: Digital citizenship is based on teaching and educating individuals in a new way - taking into account the need of these individuals for a very high level of information literacy skills. Digital Commerce: It means that the citizen is aware of how to buy and sell electronically, financial transactions through the Internet, and knowledge of e-shopping behavior.

Protection: It includes three criteria as follows: Digital Rights & Responsibilities: it means the available laws and regulations in the use of technology. Digital Health & Wellness: which means mental and physical health in the world of digital technology. Digital Security (self-protection): It means procedures for ensuring electronic safety and protection Digital security education and awareness:

- Contributing to citizens' strong Islamic education according to the Islamic faith.- Enhancing national affiliation.- Enhancing security awareness among students regarding their safety from falling into security-related crimes, establishing the principle of social responsibility and deepening the concept of comprehensive security.- Giving the individual the skill of objective and critical thinking to distinguish between correct and wrong ideas.

- Enhancing the importance of order and legal culture for citizens so as they know their rights and duties and achieve preventive security.

3. Policies and legislation: The main elements include: Identifying the sources and references of policies related to cybersecurity Preparing the main chapters of cyber security legislation and policies Building policies and legislations related to cyber security based on the main elements previously prepared and stemming from references and sources related to information and cyber security. Preparing administrative and technical regulations related to the implementation of cybersecurity policies and legislation

8- Findings and recommendations: Based on the aim of the study and the application of its methodology, the study concluded in its results that enhancing cyber awareness is based on educational training by identifying training needs related to cybersecurity and building and implementing training programs. Moreover, continuous formation on top of work is fruitful in raising awareness of cybersecurity in addition to establishing effective partnerships in the field of information culture, information and cyber security. The study also identified in its results a set of principles that contribute to raising awareness of cybersecurity through an analysis of existing and potential risks as well as indicating intended and unintended information crimes and the measurements that can be implemented about them, and how to prevent and manage crises related to cybersecurity.

The study also introduced a set of ways and procedures through which to preserve cybersecurity by presenting a set of basic security elements and means of digital culture awareness, and finally establishing measures to build policies and legislations that guarantee the preservation of cyber security. The study recommends building curricula related to information and cyber security, and before that building educational specializations to teach information and cyber security, in addition to the continuous updating of the expected risks that may occur on cybersecurity and the development of plans to face them and crisis management and prevention plans.

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Role of Varieties in the Formation of Oil-Main Raw Materials from Spring Rape Seed

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ABSTRACT

When choosing a variety for cultivation, it is necessary to take into account its genetic potential, biological features and the purpose of use. The role of ecological sustainability of varieties and hybrids is especially considerable with low availability of techno genic means.This current study mainly focuses on the role of varieties in the formation of oil-main raw materials from spring rapeseed. In fact, it discusses the yield and fat content of modern varieties and hybrids of spring rapeseed domestic and foreign breeding. To conduct this survey, field experiments and laboratory studies in 2015-2017 on the basis of Perm Gatu was carried out. The object of research was the following varieties of spring rape (*Brassica napus* L. ssp. *Oleifera annua* Metzqer): Warrior; Smilla; Is mischievous; solar CL; Mobile KL; Salsa CL; Macro; Trapper; Caliber; Akhat; Miracle.Eventually, as a conclusion, a comparative analysis of yield data showed that the domestic variety Warrior and foreign hybrids are equivalent in yield. The fat content has some variations in varieties and hybrids, but the gross collection of fat is largely dependent on the yield; that is, the formation of oilseeds per unit area is largely dependent on productivity.Having said that, another contributing factor affecting the result was the climate. For instance, based on the information acquired, the most responsive to the improvement of weather conditions over the years of research for the studied spring rape hybrids were the varieties Akhat and Smilla. All things considered, this study has attempted to investigate influential factors separately and determine their roles.

KEY WORDS: SPRING RAPE, VARIETY, HYBRID, YIELD, FAT CONTENT.

INTRODUCTION

Cultivation of adaptive, high-yielding biloba varieties and spring rape hybrids is the basis for obtaining high and sustainable yields. These varieties and hybrids can significantly improve the efficiency of oilseed production without increasing the anthropogenic load (Bome, 2000; Artyomov & Karpachev, 2005; Zhuchenko, 2005; Stepanova, and Rozhkova, 2020).

Scientists believe that the introduction in production of highly productive and adaptive varieties of spring rape can increase its yield by 25% (Korchagin, 2001; Gorshkov, 2005; Abuova, 2012; Krcek et al., 2019). Many scientists believe that hybrids are the most productive; they ripen faster and are resistant to lodging. According to Karpachova creating and introducing into production of spring rape hybrids can increase these figures by another 10-15% (Karpachova, 2008; Karpachev, 2008). According to S.I. In Astashina the yield of hybrids is 32-49% higher, and the collection of fat is 43-65% per unit area (Astashina and Astashin, 2018).

Some scientists note the advantage of foreign hybrids compared to Russian varieties. So, in the experiments of

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Wafina (Wafina et al., 2010) productivity had a hybrid of SV Sfinto. In the study of Gromova and his colleagues, the yield of the Hybrido and Siesta hybrids was 0.81 and 0.45 t/ha, respectively, higher than the variety Warrior (0.98 t/ha) (Gromova et al. 2009). The siesta hybrid was more fruitful than the Warrior variety and in the research of Gulidova and Zubkov (Gulidova and Zubkova, 2012). In the conditions of the south of the Central Black Earth region Pavlyuk (2013) recommends cultivating hybrids Siesta and Mobile KL. In extremely dry seasons, the author proposes to cultivate hybrids of Hidalgo, Mobil KL, Salsa KL and Trapper.

However, according to Seryogina, it is necessary to continue exploring hybrids due to the long period of maturation compared with domestic varieties (Sryogina 2014; Rozhkova, 2020). Thus, the introduction into the production of new varieties and hybrids of spring rape can increase the yield of both seeds and the oil content in them; increases resistance to abiotic stressful conditions, diseases and pests. However, it is essential to remember/ realize that cultivation of all varieties and hybrids is not possible in a particular area. Hence, it is preferable to consider zoned ones (Fridrihsone, 2020). Therefore, the aim of the research is to compare foreign hybrids with domestic varieties in terms of yield and oil content.

Table 1. Productivity and environmental plasticity varieties and hybrids of spring rape, t/ha oilseeds

Hybrid	Year			∑Yi	Yi	bi
	2015	2016	2017			
Warrior (k)	1.05	0.08	1.30	2.43	0.81	1.19
Smilla	1.39	0.39	2.15	3.93	1.31	1.47
Mischievous	1.13	0.62	1.03	2.78	0.93	0.51
Solar CL	2.10	0.44	0.99	3.53	1.18	1.27
Mobile KL	1.44	0.72	0.93	3.09	1.03	0.54
Salsa CL	0.72	0.61	1.26	2.59	0.86	0.39
Macro	1.56	0.70	1.13	3.39	1.13	0.73
Trapper	1.22	0.25	1.74	3.21	1.07	1.32
Caliber	1.26	0.26	1.47	2.99	1.00	1.20
Akhat	2.03	0.28	1.77	4.08	1.36	1.79
Miracle	1.79	1.00	1.23	4.02	1.34	0.59
∑Yj	15.69	5.35	15.00	36.04		
Yj	1.43	0.49	1.36			
Ij	0.33	-0.61	0.27			
NCP05	0.74	0.66	0.94		0.66	

MATERIAL AND METHODS

To solve research tasks, we conducted field experiments and laboratory studies in 2015-2017 on the basis of Perm Gatu. (Astashina and Astashin, 2018). The object of research was the following varieties of spring rape (Brassica napus L. ssp. Oleifera annua Metzqer): Warrior; Smilla; Is mischievous; solar CL; Mobile KL; Salsa CL; Macro; Trapper; Caliber; Akhat; Miracle. The repetition of the experience is threefold, and the placement of options is systematic. Accounting area plots 54 m2.Experiments were carried out on sod-fine-podzolic heavy loamy soil typical of the Middle Urals. The arable layer was characterized by medium cultivation. Agrotechnology in the experiments corresponded to the scientific system of agriculture recommended for the Middle Urals (Akmanaev, 2012; Krcek et al., 2019). The content of crude fat was determined according to GOST 13496.15-97 (GOST 13496.15-97, 1992).The significance of differences in the yield and fat content of varieties and hybrids was calculated by the method described by Armor (Armor, 2011). The ecological plasticity of varieties was compared by the method proposed by S.A. Eberhart and W.A. Russell (Eberhart & Russell, 1996).

RESULTS AND DISCUSSION

A comparative assessment of the domestic variety Warrior and foreign hybrids of spring rapeseed on the yield of oilseeds show that they are equivalent (Table 1).However, in 2015 and 2016 Miracle hybrid provided an increase of 0.74 and 0.92 t/ha of seeds, respectively, compared with Warrior. In 2015, the hybrids Solar KL and Akhat also surpassed the control variant in terms of crop yield by 2.0 and 1.9 times. In 2017, the yield of all studied variants did not differ from the standard. The best of foreign hybrids Smilla surpassed in productivity hybrids Ozorno, Solar KL, Mobile KL, Macro by 1.12; 1.16; 1.22 and 1.02 t/ha, respectively (Krcek et al., 2019). According to the data obtained, the most responsive to the improvement of weather conditions over the years of research for the studied spring rape hybrids were the varieties Akhat and Smilla. These hybrids are demanding to a high level of agricultural technology, as only in this case they will give the maximum return. In these studies, the Salsa KL, Ozorno, Mobile KL and Miracle hybrids were the least responsive to improving growing conditions (Rozhkova, 2020).

Thus, when selecting varieties and hybrids in a particular farm, it is necessary to focus not only on the level of yield of a breeding achievement, but also on its responsiveness to growing conditions. On average, over three years, the advantage in fat content was observed in the hybrid Akhat, Ozorno, Macro and Trapper (table 2). These varieties in the seeds formed more than 46% fat. The variety Warrior in all the years of research was inferior to them in this indicator. In 2015, the highest fat content of 44.2% was observed in the Macro hybrid. Somewhat less fat, 42.3% in seeds accumulated the Akhat hybrid. Hybrids Mobile KL and Salsa KL were also distinguished by a large presence of fat in the seeds. The lowest-fat content in the seeds of the 2015 crop was observed for the Solar CL hybrid. In 2016, the highest fat content was observed in the seeds of hybrids Ozorno, Solar KL and Caliber (Gains to the control were 5.6-6.6%). Somewhat lower fat content was recorded in the seeds of Smill's hybrid 41.7%, which is 0.8% more than in the variety Warrior.

The weather conditions of 2017 contributed to a greater accumulation of fat in them. The highest values of this

indicator were observed in the Trapper and Akhat hybrids (53.4 and 54.3%, respectively, which is 4.5 and 5.4% more than in the Warrior variety). The Smilla and Caliber hybrids contained the same amount of fat in their seeds as the Warrior variety. The remaining options provided an increase in this indicator from 1.2 to 4.0%. On average, for three years of studies on the gross fat collection, the studied varieties and hybrids were comparable. At the same time, trends in increasing the gross fat collection per unit area of the Smilla, Akhat and Miracle hybrids have been revealed. However, these trends have not been proved by mathematical processing and are associated with an increased fat collection from 1 ha over the years. In 2015, the highest gross yield of fat per unit area was obtained in the variants with hybrids Solar KL and Akhat, 773 and 859 kg/ha, respectively, which is 1.9-2.1 times more than the variety Warrior. In 2016, the Miracle Hybrid had the advantage, and in 2017, Smilla had the advantage.Thus, the production of oilseeds from varieties and hybrids of spring rape from a unit area is more dependent on the yield of seeds and less on the fat content in them.

Table 2. Content and gross collection of fat varieties and hybrids of spring rape

Sort	Fat content, %				Gross collection of fat, kg/ha			
	2015 year	2016 year year	2017	the average	2015 year	2016 year	2017 year	the average
Warrior (k)	38.5	40,9	48,9	42,8	405	32	637	358
Smilla	37.9	41,7	48,6	42,7	525	162	1045	577
Mischievous	39.3	46,9	52,5	46,2	445	291	541	426
Solar CL	36.9	46,5	50,3	44,6	773	203	497	491
Mobile KL	40.7	43,9	52,7	45,8	588	318	490	465
Salsa CL	40.0	46,1	50,3	45,5	289	281	635	402
Macro	44.2	45,4	50,1	46,2	690	317	564	524
Trapper	36.7	42,5	53,4	46,6	446	105	929	493
Caliber	37.9	47,5	48,7	44,7	477	124	717	439
Akhat	42,3	45,7	54,3	47,4	859	127	960	649
Miracle	38.7	46,5	52,9	46,0	693	465	652	604
NCP05	1.3	0,4	0,3	3,4	307	304	495	302

CONCLUSION

Since the role of genetic potential, biological features and climate circumstances in selecting choosing a variety for cultivation are beyond question, this study investigated the role of varieties in the formation of oil-main raw materials from spring rapeseed, including the yield and fat content of modern varieties and hybrids of spring rapeseed domestic and foreign breeding. The results based on the data obtained from the experiment conducted demonstrated that the domestic variety Warrior and foreign hybrids are equivalent in yield. Besides, the fat content has some variations in varieties and hybrids, but the gross collection of fat is dependent mainly on the yield.

Conflict of Interest: The authors declare that there is no conflict of in-terest

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Telerehabilitation in the Field of Speech Language Pathology During Pandemic Covid19 Outbreak-an Analysis in Kerala

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ABSTRACT

Telerehabilitation is the application of telecommunications technology for the delivery of speech language pathology and audiology services at a distance by linking clinician to client or clinician to clinician for assessment, intervention, and/or consultation. It is an emerging field, but due to the lack of trained professionals, the number of professionals providing telerehabilitation in Kerala is few in number. The outbreak of the pandemic COVID 19 has forced the speech language pathologists (SLPs) to shift from the traditional face to face therapy to telerehabilitation which was a new experience for most of the speech language pathologists. The present study aimed to understand the challenges faced by the speech language pathologists to provide telerehabilitation services to the clients during the outbreak of pandemic COVID-19 and how they overcome those barriers using a self- rated questionnaire developed. The questionnaire was sent to speech language pathologists through mail and WhatsApp. 105 speech language pathologists responded. Among them, 77 speech language pathologists provided telerehabilitation and served clients of all ages and different disorders with language disorder being the most common and dysphagia and apraxia being the least served client population. Telerehabilitation was found to be a viable form of service delivery in the field of speech language Pathology. All possibilities of Information and Communication Technology (ICT) were utilized by the SLPs to provide the best services despite the lack of training and non-availability of resources. This survey depicts the need for publishing standard guidelines for providing telerehabilitation services and also it emphasizes the need for improved infrastructure and training to professionals to ensure quality services to their clients.

KEY WORDS: COVID 19, KERALA, SPEECH LANGUAGE PATHOLOGY, TELEREHABILITATION.

INTRODUCTION

Corona virus disease-19 is caused by a new strain of coronavirus, which previously was referred to as '2019 novel coronavirus' or '2019-nCoV.' With the recent outbreak of pandemic COVID 19, social distancing is practiced all over the world to prevent the spread of the disease. This has led to the exploration of the possibilities of technology in almost every aspect of life. Telerehabilitation refers to the use of Information and Communication Technologies (ICT) to provide rehabilitation services to people remotely in their homes or other environments. Telepractice in speech language pathology is the application of telecommunications technology to the delivery of speech language pathology and audiology professional services at a distance by linking clinician to client or clinician to clinician for assessment, intervention, and/or consultation, (ASHA, 2005a , Brennan et.al, 2009, World Health Organization, 2020).

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The Special Group Interest (SIG) 18 of American Speech and Hearing Association (ASHA) is Telepractice. The aim of SIG 18 is to provide education, leadership, and advocacy for issues in telepractice for audiology and speech-language pathology. More than 1,000 speech-language pathologists and audiologists joined SIG 18 within a span of four years (Brown, 2014). In India, telepractice in the field of speech language pathology is only a decade old. The launch of a specialised centre for rehabilitation and education through distance mode in 2009, by the All India Institute of Speech and Hearing, Mysore may be considered as a formal beginning of teleservices.

There are only a few speech language pathologists in India providing telerehabilitation in the field of speech language pathology. The number of participants in the reported studies from India indicates this (Rao and Yashaswini, 2018; Mohan et.al, 2017). The first published article on telepractice in India was a case report of a person with Broca's Aphasia. The results showed significant improvement in the domains of expression, repetition, naming and memory. The authors concluded that telepractice is effective in the Indian context and is an upcoming area in the field of speech language pathology (Goswami, Bhutada and Jayachandran, 2012).

The first ever survey on telepractice in Speech Language Pathology and Audiology in India was carried out by Mohan et.al (2017). The questionnaire was emailed to the members of ISHA (Indian Speech and Hearing Association). There were 205 respondents out of which only 25 respondents reported using telepractice to deliver clinical services. The results showed that telepractice services were provided to clients throughout the lifespan. The service provided includes screening, assessment, management, follow-up or monitoring/ guidance and/ or professional consultation. Disorders managed via telepractice include various child and adult speech language disorders. Among the disorders, speech sound disorders in children ranked first and motor speech disorders were ranked least.

Positive feedback from clients about telepractice services was received by fifty-six percent of the tele practitioners. Lack of training was a reported drawback and a short-term training certification course in telepractice was suggested by telepractioners. The service providers in India had learned to implement telerehabilitation through personal experience rather than formal training. 92% of the tele-practitioners reported that they have not authored a publication on telepractice. This suggests a dearth of research on telerehabilitation in speech-language pathology and audiology in India (Mohan et al., 2017).

The shortage of professionals in India to deliver clinical services can be met via the use of telerehabilitation. In a study on tele-speech language pathology and audiology in India, the majority of the participants reported technical issues as barriers for telerehabilitation. Other challenges reported in the delivery of telerehabilitation were concerns about client and clinician confidentiality, lack of direct feedback and environmental distractions at client end. The benefits identified were improved access of services to clients with linguistic and cultural diversity; increased ease of collaboration among multi-disciplinary team members; and saves travel time with cost benefits for clients.

India is well equipped to fully develop telerehabilitation to overcome the barriers of distance and amplify the availability of speech language pathology, audiology and other healthcare services. The extensive use of telerehabilitation throughout India would require an improved infrastructure (e.g., to uphold privacy and security); training for professionals; and telerehabilitation policies (Rao and Yashaswini, 2018).The outbreak of the pandemic COVID 19 has forced the speech language pathologists to shift from the traditional face to face therapy to telerehabilitation which was a new experience for most of the speech language pathologists. The American Speech-Language-Hearing Association (ASHA) has mentioned that telepractice is a viable process for delivering SLP services during COVID-19 pandemic and that both evaluation and treatment were possible through telepractice.

Recently, Sarsak (2020) emphasized the need of speech language and hearing associations to promote telerehabilitation during the outbreak of COVID-19. Courses on telerehabilitation can be conducted by these associations in various countries to increase the awareness of speech therapists on telerehabilitation and also research on this issue is prioritized. It will also improve the attitude of therapists toward telepractice and update their knowledge and skills. These associations must also pursue legal efforts to make these services legitimate. Overall, it is suggested that further measures can be taken by the professional associations to eliminate barriers in the path of therapists and promote telepractice facilitators so that this type of care be used more extensively by SLPs (Tohidast et al,2020).

The present study is aimed to understand the challenges faced by the speech language pathologists to provide telerehabilitation services to the clients during the outbreak of pandemic covid 19, how they overcome those barriers, and benefits of telerehabilitation. Information gathered from this survey results can be used to upgrade the professional performance of speech language pathologists during this COVID 19 pandemic, develop the quality of telerehabilitation delivered to patients, mend existing deficiencies of the services provided through telerehabilitation and create awareness about various aspects of telerehabilitation. There is a dearth of published literature on telerehabilitation in India and there are no published studies on telerehabilitation in Kerala to the best of the authors' knowledge.

Hence the objectives of the present study were to : Report the status of telerehabilitation in speech-language pathology in Kerala, Compare the opinions of Speech-

Language pathologists about telerehabilitation in Kerala, Report the challenges faced by Speech Language Pathologist during telerehabilitation and the strategies practiced to overcome them and to identify the benefits of telerehabilitation in speech-language pathology.

MATERIAL AND METHODS

The study was conducted in three phases; development of the questionnaire, administration of the questionnaire and the analyses of the responses. In phase 1, a questionnaire was developed to gather responses from Speech Language Pathologists practicing in the state of Kerala and it consisted of 25 questions. The questionnaire contained questions to collect the demographic details of the participants and questions to elicit the information about service delivery through telerehabilitation. Twenty one closed ended questions were used to gather the opinions of SLPs about telerehabilitation. The given questions in the questionnaire addressed the opinions of Speech-Language pathologists about telerehabilitation, the challenges faced by Speech Language Pathologist during telerehabilitation, the strategies practiced to overcome them and the benefits of telerehabilitation.

Most of the questions required a response selected from multiple options and open-ended response options were given for the questions where the participants could provide their comments. The participants could select more than one response from multiple options for some of the questions and some of the questions were answered with either yes or no. The clinicians who have not provided telerehabilitation services could submit the questionnaire after filling the 8th question. Content validity of the questionnaire was done by 5 speech language pathologists and the questionnaire was modified according to the suggestions given.

Table 1. Demographic details of the participants

Demographic details		Number	Percentage
Gender	Male	4	3.8
	Female	100	96.2
Years of experience	-	-	6 months -30 years
Educational qualification	MASLP	54	51.9
	BASLP	28	26.9
	MSc SLP	12	11.5
	MSc Audiology	4	3.8
	PhD	4	3.8
	MSc Speech and Hearing	1	1
	MSc Deglutology	1	1
Work set up	Academic institute	35	33.7
	Clinics	22	21.2
	Govt hospitals	11	10.6
	Private hospitals	14	13.5
	Private practice	9	8.7
	Special school	3	2.9
	Block resource center	2	1.9
	Rehabilitation centers	6	5.8
	Urban resource centers	1	1
	NGO	1	1

In the second phase, the developed questionnaire was transformed into a google form. The google form was sent to speech language pathologists through mail and WhatsApp and 105 speech language pathologists working across the state of Kerala responded to the questionnaire. Informed consent was taken from all the participants. Analysis of the responses was done in the third phase. Descriptive statistics is used to analyse the responses from the participants. Responses were analysed by calculating the percentage values of the questions. This was done separately for each participant and also for the overall responses.

RESULTS AND DISCUSSION

The results of this study give an outline about the status of telerehabilitation in the field of Speech Language Pathology in Kerala and the results were analysed using descriptive statistics. The demographic details are given in Table 1. There were 104 respondents and years of experience ranged for 6 months to 30 years. Majority of the respondents were post graduates and were working in academic institutions, clinics and hospitals. The gender distribution was skewed towards females with 96.2% of the respondents being females. This gender disparity

in the field of speech and hearing has already been established (Rowden-Racette, 2013).

Table 2. Common reasons for not providing telerehabilitation

Reason	Percentage of respondents
No insistence from clinics or institutes	51
Client population is difficult to handle	25.9
Not confident in providing telerehabilitation	11.1
Clients or parents are not interested.	14.8

Figure 1: Percentage of respondents who delivered various services

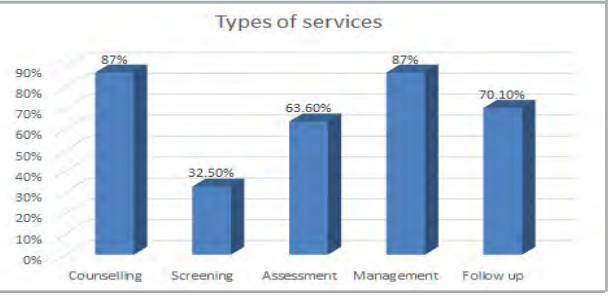
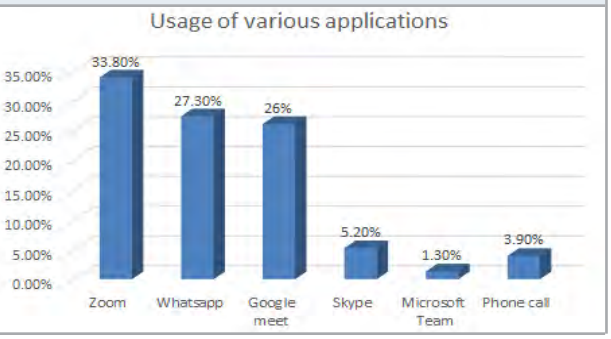


Figure 2: Percentage of users of various online platforms



The tele-rehabilitation services were provided by 74% (N=77) of the participants and 26% (N=27) of the participants did not provide telerehabilitation services during the pandemic COVID 19. The most common reasons for not providing telerehabilitation are given in Table 2. In the survey conducted by Mohan et.al (2017), there were only 25 SLPs doing telepractice in India among the 205 respondents. However, in the current survey in Kerala, 77 SLPs are providing tele-rehabilitation services among 104 participants. The lockdown followed by the pandemic Covid 19 has imposed the SLP's to shift from the traditional face to face therapy to telerehabilitation which is the best method to provide the required services during Covid 19 (Tohidast et.al, 2020).

Majority of the SLPs (79.2%) had no experience in telerehabilitation before the outbreak of Covid 19 and 20.8% (N=16) had experience in delivering telerehabilitation. Their experience in providing telerehabilitation ranged from one month to three years. Among the speech language pathologists who provided telerehabilitation, 76.6% people agree that tele rehabilitation is a viable form of service delivery, 15.6% are not sure and 7.8% did not find telerehabilitation as a viable form of service delivery. This finding is in agreement to the earlier survey conducted by Mohan et.al in 2017. Figure 1 shows the percentage of respondents providing various telerehabilitation services. Various services provided through telerehabilitation include counselling, assessment, screening, management and follow up sessions. Similar results were observed by Mohan et al (2017).

Figure 3: Percentage of clinicians who used different modes of consent.

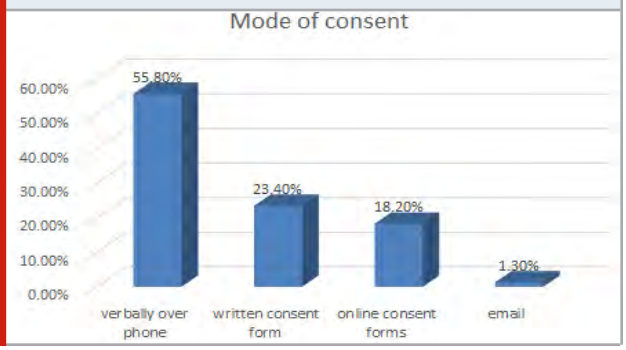
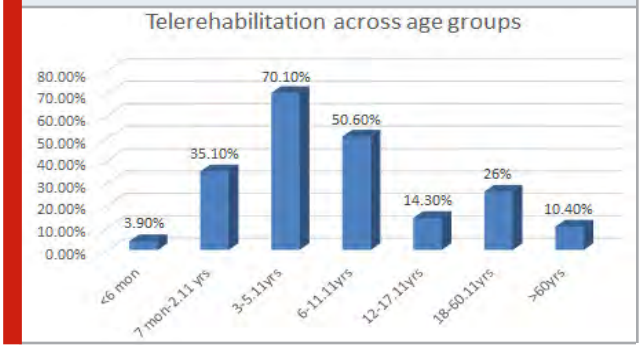


Figure 4: Percentage of clinicians providing telerehabilitation for clients across various age groups.



The various online platforms used by SLPs for delivering telerehabilitation included zoom, whatsapp, google meet, phone call, skype and microsoft teams. Percentage of use of each online platform are given in Figure 2. Two participants reported that they may use any of the above mentioned online platforms depending on the comfortability of their clients. The most reported reasons for using the specific apps were familiarity of the clients with the app (64.9%), familiarity of the clinician with the app (36.45%), better video quality (27.3%) and reduced data usage (14.3%). Initially in India, Skype was used for telerehabilitation. Over the time, many video conferencing applications were developed which were user friendly. Custom made applications were also developed by private centers for their own use, but none

of the respondents in the present survey mentioned that. In the present study, all were using the free video conferencing applications which are available over the internet. First research paper on telerehabilitation from India reported the extensive use of the skype application for telerehabilitation. (Goswami et.al, 2012).

Taking consent before the commencement of telerehabilitation is very important. Lack of ethical guidelines, issues related to privacy and confidentiality on e-platforms, data protection were issues which required immediate attention in telepractice (Rao & Yashaswini, 2018). A well written informed consent may resolve the issue to an extent. Even though the mode of getting consent was different, all SLPs except one had taken consent from the clients. This shows the awareness regarding this issue among the SLPs. Percentage of clinicians who used different modes of consent is summarised in Figure 3. The guidelines issued by ISHA clearly states the need for getting informed consent prior to the commencement of telepractice. Clients should be well informed regarding the modality of service delivery, its benefits and limitation, their rights and responsibilities including the process for communicating complaints or feedback (ISHA, 2020).

Telerehabilitation services were provided to clients of all ages. Figure 4 depicts the percentage of clinicians providing telerehabilitation for clients across various age groups. Even though telerehabilitation was provided to clients of all age groups, the paediatric population were served the most. Similar results were observed in the survey done by Mohan et.al in 2017.

Table 3. Percentage of clinicians who served various disorders served through telerehabilitation

Disorder/ client population served	Percentage of respondents
Language Disorder	53.2
Autism spectrum Disorders	46.8
ADHD	15.6
Learning disabilities	24.7
Cerebral Palsy	13
Global developmental delay	23.4
Speech Sound Disorders	41.6
Fluency disorders	39
Aphasia	23.4
Dysarthria	14.3
Voice and resonance disorders	15.6
Cognitive communicative disorders	10.4
Hearing loss	24.7
Clients attending auditory verbal therapy	10.4
Clients using AAC	9.1
Dysphagia	1.3
Verbal apraxia of speech	1.3

Through telerehabilitation, clients with various speech and language disorders were served which is summarised in Table 3. Results revealed that language disorders were the most common client population served followed by autism spectrum disorder and speech sound disorder. Dysphagia and apraxia were the least served.

Table 4 summarizes the responses of the SLPs regarding the client population which are difficult to manage. Even though the majority of the participants have provided services to children with autism spectrum disorder and ADHD, they reported that these children were the most difficult to manage client population while providing telerehabilitation. This could be due to their hyperactivity and inattention which makes it difficult to sit in front of the screen and follow clinician's instructions. The difficulty to manage dysphagia through telerehabilitation was reported by only one participant and this can be due to the smaller number of SLPs providing telerehabilitation for dysphagia clients. Managing dysphagia through tele mode may involve many risks. But through proper planning and training of both client and the caregiver, we can successfully treat dysphagia through telerehabilitation. Considerations for the management of dysphagia through telerehabilitation is given by Miles et al (2020) But both research and clinical practice in this area show that the use of telehealth for dysphagia management can be safe, feasible, and reliable, but several safeguards and considerations need to be in place (Miles et al, 2020).

Table 4. Difficult to manage client population

Difficult to handle population	Percentage of respondents
Language Disorder	6.5
Autism spectrum Disorders	66.2
ADHD	51.9
Learning disabilities	1.3
Cerebral Palsy	20.8
Global developmental delay	22.1
Speech Sound Disorders	9.1
Fluency disorders	6.5
Aphasia	9.1
Dysarthria	6.5
Voice and resonance disorders	11.7
Cognitive communicative disorders	13
Clients attending auditory verbal therapy	23.4
Clients using AAC	16.9
Dysphagia	2.6

The sudden trend in telerehabilitation has led SLPs to face various challenges / barriers. Majority of the respondents did not have any previous experience in telerehabilitation and hence had to face many challenges while implementing telerehabilitation. Table 5 summarizes the client/ clinician related challenges

while providing telerehabilitation. Difficult to manage children through online mode was the one of the biggest challenges faced by SLPs followed by scarcity of online resources about telerehabilitation and non-willingness of parents or patients to shift from traditional face to face therapy to telerehabilitation.

Table 5. Client/ clinician related challenges while providing telerehabilitation

Challenges/ Barriers faced	Percentage of respondents
Lack of training	22.1
Lack of online resources	46.8
Lack of confidence	5.2
Parents or patients are not willing to attend	44.2
Difficult to manage children through online mode	76.6
Non availability of caretaker or parent	18.2
Non cooperative parents	27.3
Children with attention issues	22.1
Others	7.8

Table 6 represents the technical challenges faced by SLPs while implementing telerehabilitation. Among the technical challenges faced, internet connectivity issues stand first followed by inadequate knowledge of clients to use tele-service applications and non-availability of smartphones or computers. SLPs learned through personal experiences rather than formal training. Webinars have become a trend in the Covid season and webinars on telepractice is the only formal kind of information gaining that has happened. Similar results including insufficient resources (i.e., structural framework, technical support, resource materials) to provide appropriate tele-speech-language pathology services and the lack of formal training in India were the major concerns reported by Mohan et.al (2017).

Table 6. Technical challenges

Technical challenges	Percentage of respondents
Internet connectivity issues	94.8
No internet connection	7.8
Non availability of smart phone / computer	39
Clients not good at using applications	58.4
Non availability of interactive software	1.3

Even though the participants reported of various challenges faced while providing telerehabilitation, evident effort has also been taken to overcome these challenges which is given in Table 7. The reported solutions tried to overcome the challenges were discussing with the SLPs who have experience in telerehabilitation, trial and error method, attending webinars and reading articles on improving telerehabilitation. Development of professional skills for telepractice, validation of digital resources in the different languages of India; empirical studies on mode of service delivery in telepractice (face-to-face, virtual or hybrid); mechanisms to protect client's privacy on e-platforms; and revision of code of ethics for speech-language pathologists and audiologists who are using telepractice were the immediate concerns as reported by Rao & Yashaswini (2018) on their report on tele speech language pathology and audiology in India and these concerns still remain the same. But due to the sudden boom in telerehabilitation, we expect a sudden growth in these areas and in the near future, we will be able to overcome the barriers faced. Despite these challenges, telerehabilitation has a critical role during the infectious pandemics and it will reduce the risk of spreading the infection which are transmitted by person-to-person contact (Smith, Thomas, Snoswell, Haydon, Mehrotra, Clemmensen & Caffery, 2020).

Table 7. Steps taken to overcome the challenges

Steps taken	Percentage of respondents
Discussed with SLPs who have experience in telerehabilitation	71.4
Read articles on telerehabilitation	49.4
Attended webinars on telerehabilitation	61
Trial and error method	71.4
Educating parents	1.3
Watched youtube videos on telerehabilitation	1.3

Table 8. Benefits identified through telerehabilitation

Benefits	Percentage of respondents
Regularity in taking sessions	54.5
Increased patient access or reach	53.2
Eliminating long distance travel and thus avoiding the risk of Covid 19	92.2
Parents could involve more in therapy	61
Patient satisfaction	22.1
Easy follow up/ monitoring	46.8
Clients are comfortable in performing activities at the home set-up.	84.5
Easy to perform creative screen-based activities	1.3

There were many benefits experienced through telerehabilitation. Table 8 shows the various benefits of telerehabilitation identified by participants. Eliminating

long distance travelling thus avoiding the risk of Covid 19 was the benefit cited by majority of the SLPs (92.2%). Other benefits include comfortability of clients in performing activities at the home set-up, increased involvement of parents in therapy, regularity in taking sessions, increased patient reach or access, easy to follow up, easy to perform creative screen-based activities and patient satisfaction. The benefits of telerehabilitation makes it a viable form of providing services to clients with speech and language impairments and the majority of clinicians could achieve their goals through telerehabilitation. The technological advancements and the benefits can make it a regular form of service even after the COVID 19 season. The scarcity of resources and other issues such as lack of legal guidelines and policies for safe and secure service delivery should be addressed by the concerned authorities at the earliest. Recently Indian Speech and Hearing Association (ISHA) has compiled and published the telepractice guidelines for audiology and speech language pathology services in India including operational and ethical aspects (ISHA, 2020).

Figure 5: Methods of documentation used by SLPs

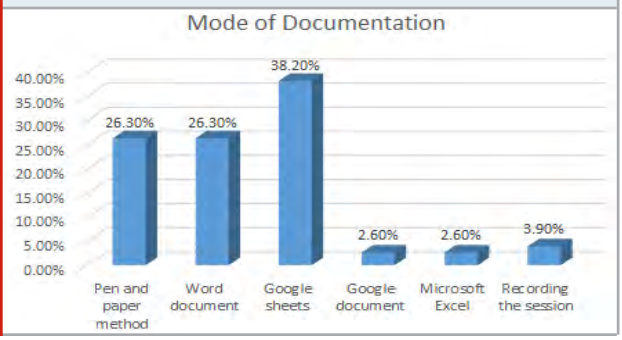
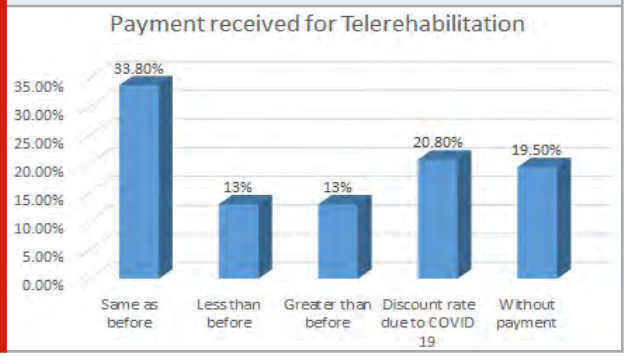


Figure 6: Feedback from clients

Different methods of documentation used by SLPs were depicted in figure 5. As noted from the chart, the various methods include traditional pen and paper method, word document, google sheets, excel sheets, google docs and recording of sessions were reported. The clinical record is an overall indicator of clinical and service quality, and serves as a basis for planning care and for service continuity (Sutherland, 2006). Documentation style may vary among professionals or organizations but should adhere to specific facility standards. Whatever be the style, clinical records should be consistent in format and style and should use appropriate terminology, approved abbreviations, and correct diagnosis and procedure codes. Majority of the respondents in the survey use various electronic documentation methods. E- records give the flexibility of accessing it anywhere, anytime. Indian Speech Hearing Association had recommended storing all the client related reports and records of the telepractice session using the unique patient identification number in a confidential manner (ISHA, 2020).

Majority of the tele practitioners could achieve the targeted goals through telerehabilitation. 74% (N= 57) of the tele practitioners reported that they could achieve the goals through telerehabilitation. 22.1 % (N=17) were not sure and 3.9 % (N=3) reported that they could not achieve the goals. A small percentage who could not achieve their goals would be those tele-practioners who serve the difficult to handle population such as clients with dysphagia, autism spectrum disorder or ADHD. The feedback from the clients regarding telerehabilitation as reported by the SLPs is shown in Figure 6 and only one SLP among the participants reported that the clients were not satisfied with telerehabilitation.

Figure 7: Payment received for telerehabilitation



The payment received for telerehabilitation services were differing among SLP's as depicted in Figure 7. To the best of authors' knowledge, telerehabilitation services are charged more than the traditional face to face therapy due to the use of high-speed internet data and the increased time required for planning and preparation. But in the present study, only a small percentage (13%) of SLPs have charged more than the charge paid for traditional face to face therapy. Majority (33.8%) has charged the same fees as that of pre COVID face to face therapy. Some provided telerehabilitation services at a discounted rate (20.80%) due to the COVID 19 and some had provided services free of cost (19.50%).

Free telerehabilitation services were provided by SLPs working in government services including hospitals and institutes. To the best of authors' knowledge, none of the previous studies had mentioned the fees charged for telerehabilitation. Regardless of the fees charged, telerehabilitation is the best method to provide the required services during Covid 19 (Tohidast et al, 2020). To fulfil the need for continuous therapy sessions for children and adults with speech-language disorders, the

implementation of telepractice in the field of Speech Language Pathology is necessary which will also help to prevent the transmission of COVID-19, and thereby guaranteeing the health of SLPs and patients (Smith et al, 2020).

CONCLUSION

The present survey was conducted using a self-rated questionnaire and reported the status of telerehabilitation in the field of speech-language pathology in Kerala, the challenges faced by Speech Language Pathologist during telerehabilitation, the strategies practiced to overcome them and the benefits of telerehabilitation in speech-language pathology. Even though all of the participants were practicing traditional face to face therapy before the outbreak of COVID 19 pandemic, majority of them easily shifted to a tele-mode of providing services during the COVID 19 pandemic outbreak. This survey depicted the need for publishing standard guidelines for providing telerehabilitation services. At the time of data collection, there were no published standard guidelines in India for providing telerehabilitation. But in November 2020, Indian Speech and Hearing Association (ISHA) came forward with the guidelines for telepractice which will help the telerehabilitation service providers to provide better services to their clients. It also emphasizes the need for improved infrastructure and training to professionals to ensure quality services to their clients.

Conflict of Interest Statement: There is no conflict of interest to disclose.

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Framework to Build up a Business Model for Content Creation Using Information Technology

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ABSTRACT

Present research project has proposed framework for the content creation by use of IT technology. The framework for content creation using technology would utilize tools such as learning management systems, online enabled classrooms, management information systems and e-learning. In the Gulf, increasing online learning platforms will bring alignment, productivity, value, remote accessibility, plasticity, and access to information with simplicity and ease of studying especially in the current global crisis that has been occasioned by COVID-19. The framework to facilitate online learning would enable working people, students with family commitments and mobile people such as refugees' access quality education. Coming up with the framework has been based on the systematic literature review results.

KEY WORDS: FRAMEWORK; GULF; CONTENT-CREATION; INFORMATION TECHNOLOGY; LEARNER.

INTRODUCTION

Technology has transformed almost every aspect of life in the current times. The impact of Information and Communication Technology (ICT) in the age of globalization and in the information age is apparent in learning institutions especially in the higher education institutions such as universities (Alzahrani, 2017). Particularly, the use of platforms for content creation as enabled by the access to internet and availability of digital gadgets especially for the youthful population has improved learning and information sharing approaches. In the developed world, ICT has greatly changed how classroom learning and also how teaching is conducted. Additionally, it has facilitated diverse programs such as distance learning and enabled greater access to learning materials especially through digitized libraries (Duangekanong and Vate-U-Lan, 2019).

In the Gulf region however, online platforms have not been fully used by universities to create and provide content since face to face learning is the favored learning method (Salloum et. al, 2019). A framework to build a business model for content creation and sharing would necessitate access to a digital platform that would utilize technology to ensure that end users be they faculty members or student's access relevant information (Al Tamimi, 2017). This is useful in the current crisis brought about by COVID-19, which has resulted in governments requesting citizens to practice social distancing and also limit their interaction by staying at home to avoid transmission of the virus (Alandijany et al., 2020).

Many institutions of higher learning have modernized their business model from the traditional brick and mortar to a business model of bricks and clicks. This has resulted in these institutions developing online platforms to provide courses to their students and content for faculties to facilitate their teaching (Bayne and Jandric, 2017).

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For example in the UK, it is estimated that ninety five percent of higher learning institutions have adopted the online learning business model and that online courses have increased access to learning content for student as one in three student now takes at least one course online during their college life resulting in reduced student fees by sixty five percent (Allen and Seaman, 2017). Jin and Cortazzi (2017) believe that technology is the solution to most of the challenges in the academic field in this era of globalization especially in the Gulf region.

A framework for content creation using technology would utilize tools such as learning management systems, online enabled classrooms, management information systems and e-learning. Integrating these ICT platforms has been found to be useful to support universities in the system of content creation (Rodrigues et al., 2019), to support students acquire critical career and soft skills and also assist in bridging the distance between the lecturers, students and institution's management (Alzahrani, 2017; Al-Kindi and Al-Suqri, 2017). The framework is to facilitate online learning would enable working people, students with family commitments and mobile people such as refugees' access quality education (Rostron, 2018). In the Gulf, increasing online learning platforms will bring alignment, productivity, value, remote accessibility, plasticity, and access to information with simplicity and ease of studying especially in the current global crisis that has been occasioned by COVID-19 (Alandijany et al., 2020).

Research Problem: Content creation business models in the United Kingdom and other developed countries in the world are based on implementation of evolving technologies and innovative and up to date techniques in education (Bayne and Jandric, 2017). There are many technologies that encourage interaction between students, faculty members or among students themselves (Yulisman, 2017). Studies carried out agree that online learning and teaching contribute to various benefits but require resolving barriers to development, uptake, advancement of new skills and learning approaches, and increasing commitment to the stakeholders. Online learning offers many benefits and appeals to the upcoming generation of youthful students (Al-Kindi and Al-Suqri, 2017). Institutions of higher learning in the Gulf have embraced a number of technologically enabled content sharing practices such as the use of YouTube for instructive videos and content sharing, audio-visual lectures, delivery of instant feedback to learners, online transmission of assignments and ensuring assignments are automatically graded (Shah, 2017).

However, the adoption in the Gulf of these business models by universities that utilize online content creation and sharing has been slow in uptake (Salloum et al., 2019). Most universities that have adopted these modes of content sharing have been motivated by international collaborations (Salehi-Isfahani et. al, 2018). As a result, these models have adopted a lot of the practices from universities in the developed countries such as the United Kingdom. There is also a high population of youthful population getting into universities who are tech-savvy and who would benefit most from the online learning platforms. Faculty readiness has been seen to be a major barrier to development of this business model that uses technology for content creation and provision (Salloum et al., 2019).

Due to the adoption of western frameworks in some of the institutions that have adopted digital content sharing in the Gulf, there is need for a framework that develops business models that take into account the uniqueness of the Gulf region (Al-Tamimi, 2017). Additionally, there is need to capture a larger audience such as the youthful population and other remote persons such as caregivers who are committed to their families but who would like to pursue and education (Rostron, 2018). In the current COVID-19 crisis that has seen governments in the Gulf region call for increased social distancing and other self-isolation measures (Alandijany et al., 2020), there is need for the higher learning institutions to develop a framework for digital platforms to facilitate continued remote classroom learning and knowledge sharing in institutions of higher learning (Assaad et. al. 2020).

To realize this, the present study efficiently responds to two research questions: What are the current studies about IT and content creation considering the Gulf viewpoint? What framework, founded on IT and content creation, can be applied to build a business model? Significance of the Study : This research on a framework to build up a business model for content creation using IT technology significant since there are few research projects that focus on similar frameworks especially with a focus on the Gulf region. Previously, researchers have shown that higher learning institutions that have developed a business model that utilizes technology to create and share content are more efficient and effective in academic activities (Al Tamimi, 2017). The current research will be insightful to higher learning institutions based in the Gulf. Further to this, this study is significant, as it will reduce the gap that currently exists in the current body of study due to lack of research on comparable matters.

Limitation of the Study: The methodology used in this research project forms an important part of the limitations. A systematic literature review (SLR) was applied in this research (Xiao & Watson, 2019). The SLR provided convenient and impartial outlines of previous studies about research topic and has assisted in overcoming the inapplicability of collecting primary data in the current COVID-19 crisis in the Gulf region (Boelens et al., 2017). Nonetheless, the disadvantage of depending on the systematic literature review methodology is that the investigator did not realize the advantages of gathering primary data such as control over the research process (Xiao & Watson, 2019), or access to the most relevant data in the prevailing crisis of COVID-19 as would have been facilitated by primary research process.

Operational Definition: The operational definition used in the current study is succinct, comprehensive and a

clear description of the method employed in the study. To have a strong research project, it is essential towards the collection of the most relevant data. The operational definition is particularly important, as the researcher has to make decisions whether data obtained is useful or impractical to reduce potential ambiguity as well as misperception. For instance, the process to obtain data would not be effective if the definition of timely and applicable data is omitted from the research (Hibberd, 2019).

To ensure effective data collection, the investigator applied a constant approach in finding and gathering the data required in the study. This involved defining how data would be gathered to eliminate the likelihood of unreliable and flawed data. By ensuring that the operational definition was comprehensive, the researcher was able to eliminate any inconsistency in data gathering for the study (Hibberd, 2019). Below is a list of operational definitions used in the study.

Characteristic of interest: Framework to build up a business model for content creation using it technology Measuring instrument: Data collected by the researcher from current technical sources that are online and from sources in the physical library.

Test method: The investigator gathered and evaluated data from a minimum of 30 scientific sources. The investigator also relied on current sources of data that were printed after 2017. Decision criteria: Thoroughly and sequentially evaluating the data obtained from various diverse sources. Recent data published after the year 2017 was relied on for the current study by the investigator. Data from unpublished sources such as manuscripts or data from material published prior to the year 2017 was excluded in the study. Previous Studies: Today the organizations in Gulf, irrespective of sectors that such organizations are operating from, have faced various emerging issues such as new technologies, new ways of doing business and most recently, a disruptor has been the Covid-19 pandemic (Alandijany et al, 2020). At same time, this has generated business challenges and opportunities for the Gulf businesses (Ebrahim et al., 2020).

As such, in light of changes in the environment and disruptions, the focus of such organizations on new business models offers an approach to surmounting the challenges and taking advantage of new opportunities, and in turn, this influences the marketing and business structures (Woertz, 2020). In that sense, a number of studies have pointed out changes offered by the information technology (IT) positively reflect on the businesses' performance, making it possible to achieve competitive advantages via innovation, and subsequently, be distinct with respect to the competition (Alandijany et al., 2020). As such, in light of market disruptors like recent Covid-19 pandemic, organizational transformation can benefit from being agile in response to some new technologies, offering development of some new ways for creating market value via innovation process, that expands capabilities of the organization and consequently result to generation of some novel business models (Salloum et al., 2019).

Nonetheless, aside from possibility of innovating in offering its products and its services, the entity needs to show concern with adequacy of its business models with respect to new upcoming technologies (Woertz, 2020). The reason for this is that recognizing threats or possibilities from such new technologies that have been introduced on market for existing business model can enable the entity reacts through realigning its services or products, logical forms, skills, processes and network relationships. This is because essence of the business model permits the organization to react, and deliver better value for its users (Farhan et al., 2019).

However, not all organizations have understood the need for adapting their current business models to the emerging changes and disruptions in the market. This is especially so in the case of educational institutions, which are more comfortable with tried and tested business models that have always worked for them over the years rather than attempting to try out an untested business model (Al Tamimi, 2017). In such instances, authors like Shah (2017) have pointed out that the competition amongst the learning institutions would not only happen through the courses offered, but would also happen via having business models that are innovative, as the innovation of the business model has a high potential of strongly impacting a market and competition (Woertz, 2020), and could make it possible for the learning institution to set up some competitive advantages. In that context, disruptive business model arises at the stage whereby the emerging innovations and technologies have become highly critical, needing new forms of organizational structure to services and products offered, emphasizing proposal of unique value to market concerned, replacing current business models (Yulisman, 2017).

In light of the recent disruptions to the higher education, review of the existing studies to understand the state of the art has presented the perspective that today, there has some little research seeking to present business models that assist in setting up systems which collect all content that higher education requires, for dealing with the emerging situations today, and using information technology (IT) in the process (Bukamal & Mirza, 2017; Engin & McKeown, 2017). Despite the prevailing perception in the Gulf region, the online platforms have no negative impact on education excellence but have assisted universities advance skills of their members (faculty and students) (Al-Kindi and Al-Suqri, 2017).

In the Gulf, increasing online learning platforms will bring alignment, productivity, value, remote accessibility, plasticity, and access to information with simplicity and ease of studying especially in the current global crisis that has been occasioned by COVID-19 9Al Tamimi, 2017). Many institutions of higher learning have modernized their business model from the traditional brick and

mortar to a business model of bricks and clicks. This has resulted in these institutions developing online platforms to provide courses to their students and content for faculties to facilitate their teaching (Bayne and Jandric, 2017).As such, bearing in mind that state of the art on the business models that assist in setting up systems that collect all content that higher education requires is just emerging and therefore lacks theoretical foundation to support it.

MATERIAL AND METHODS

Study Methodology: A systematic literature review (SLR) was applied in this research (Xiao &tWatson, 2019). The SLR provided impartial outlines of previous studies about research topic. As a first step in the SLR, the researcher came up with an initial inclusion and elimination criteria following discussions with an acquaintance. Studies associated with frameworks to build up a business model for content creation using IT technology were aggregated, reviewed and evaluated while using pre-specified and homogenous procedures. The reviewed studies met a given criteria of being about IT technologies and also about the frameworks that support the creation of business models for content creation. This method was beneficial as it assisted in narrowing down on the relevance of the sources in with regards to the study. For instance, frameworks that build a business model for content creation that was not IT based was not considered in the SLR. The researcher used particular keywords and relied on various databases to collect the information needed in the research (Xiao & Watson, 2019).

The keywords used were Information Technology; Frameworks; Business Model; Content Creation, and their synonyms. This formed the criteria for inclusion. Another criteria relied on was linked to the time period whereby only the studies carried out post 2015 were considered. This study considered the literature of nearly sixty online sources to determine their relevance on IT and based on business model for content creation. Scrutiny of the abstracts resulted in the elimination of the studies, which did not include the findings and their corresponding methodology in the examined abstracts. As a result, about twenty seven sources were considered not appropriate for the current study (Fisch & Block, 2018).

Following the initial assessment of the inclusion and exclusion criteria, the evaluation of the entire content of the studies that met the inclusion criteria ensued. Characterization of the findings in the studies was analyzed and presented in a summarized form in order to contribute to the research questions presented in this study. Patterns identified in the studies evaluated were presented in a qualitative narration (Xiao & Watson, 2019). The findings arrived at by the researcher were found to be reliable since the researcher relied on studies that has strong methodologies (quality), were recent and relevant. Owing the number of studies screened by the researcher end to end, a great level of depth and credibility was achieved throughout the process. From a focus on sensitivity of the abstract in the initial screening, the researcher focused on specificity of the entire studies with an emphasis on methodologies applied in order to ensure that the findings would be reliable as well as of good quality (Fisch & Block, 2018). Analysis of the full text led to further exclusion of studies from the SLR due to factors such as grammatical errors as well weak methodologies applied in studies.

The university's library database was the primary source of the online studies considered in the systematic literature review. Reliance on online sources as opposed to other search options such as physically going through the library the periodicals was convenient as well as expeditious. The online searches in contrast to physical ones allowed the investigator access to a wide array of current studies (Snyder, 2019). Process applied to arrive at conclusion: To ensure adequacy of findings, the researcher covered the studies precisely and thoroughly. Comparisons and contradictions of the findings in the studies reviewed were identified and evaluated in order to detect bias and to minimize the chance of perpetuating the partiality in the current study. Summaries of pertinent data from the relevant studied were input in worksheets, which were about twenty five in number.

Subsequent to the summaries being categorized appropriately, further reading by the researcher resulted in patterns being identified. Patterns in the research that were frequent were coded. The codes were categorized broadly into groups hereafter referred to as findings/ results. These results were incorporated into the current study to support the research question on the frameworks to build up a business model for content creation using IT technology (Fisch & Block, 2018). Conclusions were drawn from the findings through critically analysis and through associating and providing converging views to these findings. Research ethics was adhered to ensure impartiality of the conclusions. This was achieved through ensuring participants in the prior primary research stayed anonymous and through the researcher relying on a thorough methodology which had clarity on procedures applied to reach the conclusions presented (Snyder, 2019).

RESULTS AND DISCUSSION

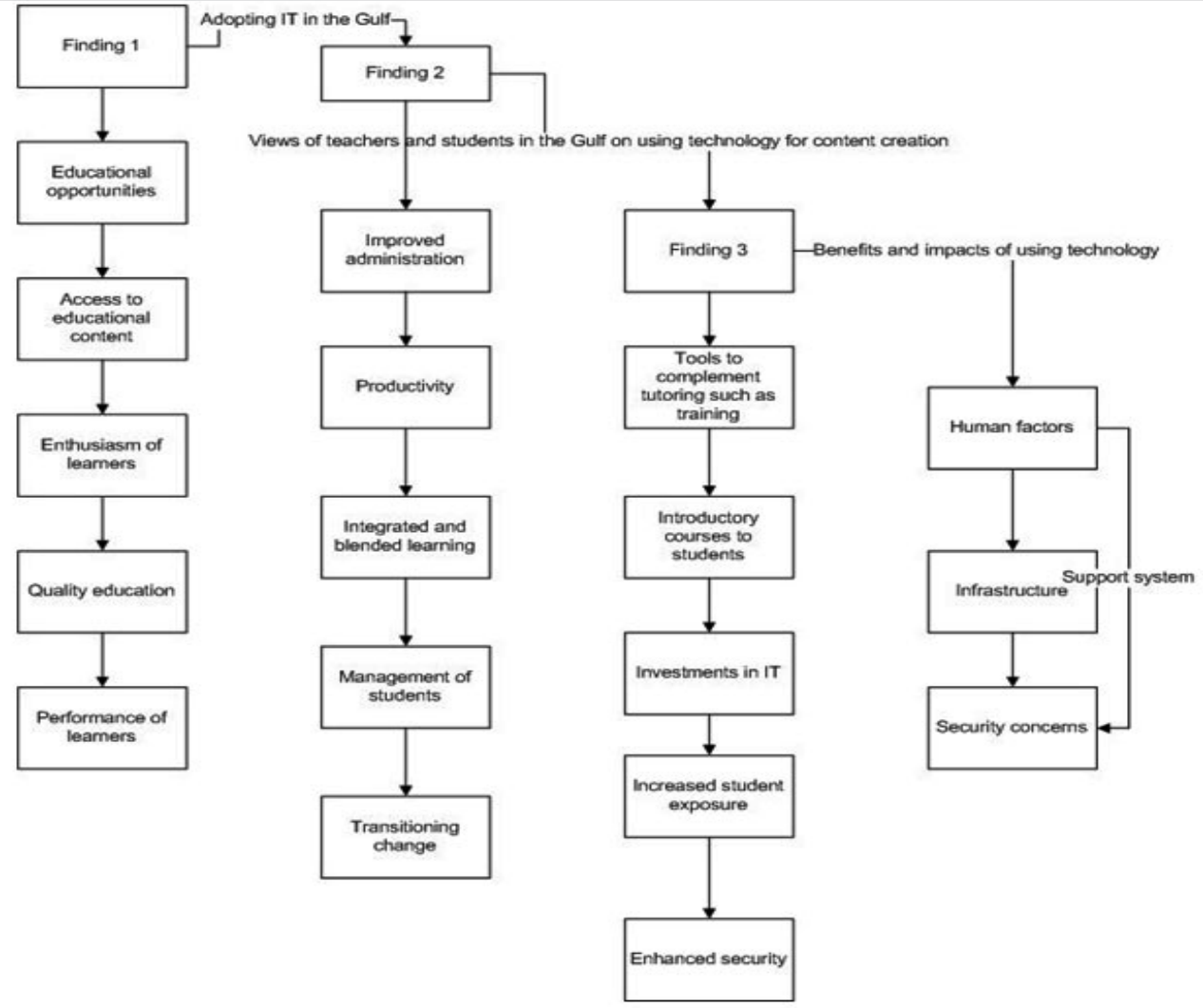
Adoption of IT technologies for building a model that can be used by higher education institutions for academic purposes in light of the changes in their environment, such as the Covid-19 pandemic, is important for improving the learning outcomes of the Gulf learning institutions, as well as improving the learning engagement and experiences of the Gulf students using IT technologies. The systematic review of the literature revealed a number of findings:

Theme 1: IT technologies, especially those relying on Web 2.0 and Internet of Things (IOT) can improve easy access to the educational content by the student, delivering some quality education, enhancing the learning opportunities as well as motivating the Gulf

learners to learn. This can enhance the learning even in the midst of the Covid-19 crisis. IT technologies in the Gulf higher learning institutions, and the content that is enabled, can be observed to be supportive 9Alajmi & Rorissa, 2018; Baadel; et al., 2017). However, the IT technology only serves as a complementary tool for facilitating administration processes and quality education. It is hard to replace traditional approach to learning, which is the face to face approach. Review of studies has demonstrated importance of the physical contact, in much the same way that complementary IT technologies helps the higher learning institution overcome some of the challenges brought about by the Covid-19 crisis (Yulisman, 2017; Shah, 2017; Farhan et al., 2019).

Theme 2: There could be negative outcomes with respect to using IT technologies for the content in Gulf higher education. In the western countries, the best practices revolve around security and privacy of data (Nouby & Alkhazali, 2017; Alajmi et al., 2018). In the Gulf, these have to be addressed or otherwise this could hinder successful implementation of the planned framework. Other issues revolve around investments in IT, and concerns over inadequate student exposure as well as restrictions based on university policy. Such concerns can potentially act as hindrance against successfully adopting IT technologies for content creation in Gulf learning institutions (Frahan et al., 2019; Al-Kindi and Al-Suqri, 2017).

Theme 3: Another observation from the systematic literature review is that in many of the developed western countries, educators have opportunities of attending training, seminars and workshops in a structured way, where they use the IT technologies for instructing the learners (Toufaily et al., 2018; David et al., 2017). Moreover, the learners do not lag behind, but rather have introductory courses where they are trained on using such technologies. This is an issue that can as well be replicated in Gulf countries (Al Tamimi, 2017; Alzahrani, 2017).



Source: Author's own

Recommendations: A framework is recommended, which consolidates the outcomes of the systematic literature review. The framework further advances better and wider consolidation of the systematic literature review findings, and enhances exactness of the trustworthiness and inferences. The framework is demonstrated in figure 1 below:

The framework for content creation using technology would utilize tools such as learning management systems, online enabled classrooms, management information systems and e-learning. Integrating these ICT platforms has been found to be useful to support universities in the system of content creation, to support students acquire critical career and soft skills and also assist in bridging the distance between the lecturers, students and institution's management. The use of platforms for content creation as enabled by the access to internet and availability of digital gadgets especially for the youthful population has improved learning and information sharing approaches.

In the developed world, ICT has greatly changed how classroom learning and also how teaching is conducted. Coming up with the framework has been based on the systematic literature review results. However it is noteworthy that such a framework cannot act as framework that is 'one size fits all' in Gulf; rather the framework offers guidance to adopting IT technologies for learning in Gulf, and for overcoming the challenges brought about by Covid-19. Moreover, the framework is largely hypothetical in nature and lacks empirical backing, despite contributing top state of art.

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Evaluation of the Accuracy of a Novel Method to Locate the Extraoral Point for the Centre of Resistance of Maxillary Dentition

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ABSTRACT

Headgears for class II correction in growing children require determination of centre of resistance of maxilla and maxillary dentition accurately. No standard accurate method is available in literature. The objective of this study was to formulate an indigenous simple method to locate the centre of resistance (C_{res}) of maxillary dentition extraorally (eoc_{res}) on the cheek and to determine whether the point marked extraorally coincides with the centre of resistance of the maxillary dentition (io_{cres}) established previously between roots of premolars radiographically. 14 patients without gingival problems seeking Orthodontic treatment were included in this study. A divider tool was indigenously designed to mark a point extraorally on the cheek which coincided with the intraoral point between the roots of the two maxillary premolars, that is believed to be the Cres of the maxillary dentition. Radiopaque gutta percha was stabilized using adhesive transparent tape on the point marked extraorally and an Orthopantomogram was taken to evaluate the position of this point radiographically. The point was located between the roots of the maxillary first molar and second premolar at a mean height of 6.8+/- 0.52 and 6.8 +/- 0.48 respectively in the OPG. It can be concluded that by using a simple divider tool the extraoral C_{res} of the maxillary dentition can be marked conveniently and accurately. Thus, this novel simple method is a clinical innovation and can be used to determine the centre of resistance of the maxillary dentition extraorally for Class II patients requiring headgears, or bodily distilization intrusion of maxillary arch.

KEY WORDS: CENTRE OF RESISTANCE; DISTALIZATION; HEADGEARS; MAXILLARY DENTITION.

INTRODUCTION

Overtime, numerous experimental methods have brought into light the concept that tooth movement is affected by various factors like magnitude, direction and the duration of the forces applied, direction of force playing the most pivotal part, (Tanne and Sakuda 1991, Burstone 2015; Eliades et al. 2017). Hence, this urged the interest in biomechanics to study the overall effect of extra oral traction devices to the applied forces. The understanding of the relationship of the force vector to the centre of resistance (C_{res}) of the dentition became vital to appreciate the mechanical properties of the extraoral traction appliances. Centre of resistance is analogous to the centre of mass for restrained bodies i.e. tooth within the restrained boundaries of the periodontal ligament. In previous studies, C_{res} of a tooth or group of teeth or an entire dentition revealed that an important factor in tooth displacement is the point of application of force and the location of C_{res} of the maxillary complex and dentition

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offers integral information with respect to the usage of headgears, (Kragt and Duterloo 1983; Duterloo et al. 1985, Bulcke et al. 1987 Stocker et al 2020).

Extraoral traction forces applied via headgears have been used in many years to completely restrain or redirect the growth of maxilla in Class II malocclusions. (Stocker, Patcas, and Schätzle 2020) (Papageorgiou et al. 2017). Headgears has numerous applications like anchorage control, distalization of molars (Sadeghi, Hedayati, and Mousavi-Fard 2019, Alosman, Bayome, and Vahdettin 2020), restrict maxillary growth, simultaneous distillation of premolars and molars, (Biswas 2008) , complete distal displacement of the maxillary complex and maxillary dentition (Deguchi et al. 2008). Commonly encountered adverse effects due to ignorant biomechanics are extrusion and tipping of molar teeth (Rosa et al. 2020) which may cause the mandible to rotate posteriorly causing facial elongation and affecting the function and esthetics.

Force is delivered to the teeth by means of a face-bow attached to an intraoral bow which is placed into buccal tubes on the molar bands or by direct attachment of the arms of headgear on the maxillary arch wire itself by means of hook. The outer bow may be short of the C_{res} (flattens the occlusal plane) or long (steepens the occlusal plane) or passing through the (C_{res}) (no change in the cant of occlusal plane) of the maxillary complex and dentition and coincide with the position of the inner bow to produce a translatory distillation force devoid of any untoward movements (Greenspan 1970). Thus, it is essential to accurately determine the (C_{res}) of the maxillary complex and maxillary dentition.

No accurate and standard method has been established in literature to locate the C_{res}. Thus, the objective of this study was to formulate an indigenous simple method to locate the centre of resistance of maxillary dentition extraorally on the cheek and to determine whether the point marked extraorally on the cheek coincides with the centre of resistance of the maxillary dentition established previously between roots of premolars radiographically.

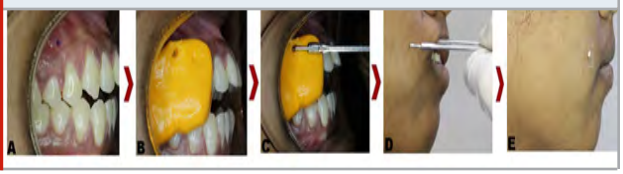
MATERIAL AND METHODS

This was a pilot study conducted on 14 patients in the age group of 14-40 years reporting to the Department of Orthodontics in a private hospital seeking treatment. The routine clinical examination and diagnosis was done for the patients. The methodology which was employed for this study was as follows: (Figure 1 and 2).The patients were seated on the dental chair and were asked to look straight at their eye levels.. The area near the roots of the 1st and 2nd maxillary premolars was isolated and a blue point was marked between them at distance of 10 mm from the Cementoenamel l junction i.e intraoral centre of resistance (ioc_{res}). This point was determined based on the known facts that the approximate root length of both premolars is 14 mm (Nelson and Ash, 2010) and the centre of resistance is generally 1/3rd to 1/4th the distance from the root apex (Bulcke et al., 1987). Putty was placed on occlusal surfaces of the premolars and pushed apically upto the mark and the patient was asked to bite gently.

The indigenous divider was then taken and the arm which was bent at right angles was passed through and through the putty prior to its set to make sure it coincided with the point marked between the two premolars. This arm was stabilized and the putty was allowed to set. Once the putty set (3.5 minutes; Zhermack Elite HD+ company), the soft tissues were relaxed and the other arm of the divider was closed and approximated extraorally on the cheek. This point was marked with blue marker and a small piece of the 6% gutta percha was cut and placed on the point and stabilized with transparent adhesive tape .Orthopantomograms (OPG's) were taken for each patient with the gutta percha point in place as routine diagnostic X-rays prior to treatment without exposing them to any additional radiation. Gutta percha was used specifically in an attempt to use a radiopaque material which would be distinguishable on the radiograph. Once the OPG's were obtained, the point could be identified as a radiopaque point. The linear distances from the radiopaque point to the edges of roots of the premolars and molars, and the distance between the CEJ to the point was made and mean values were obtained by using FACAD software. (Figure 3).

Figure 1: Armamentarium: From left to right: An indigenous divider tool, marker, 6% gutta percha point, putty material.

Table 1. Methodology to mark the centre of resistance

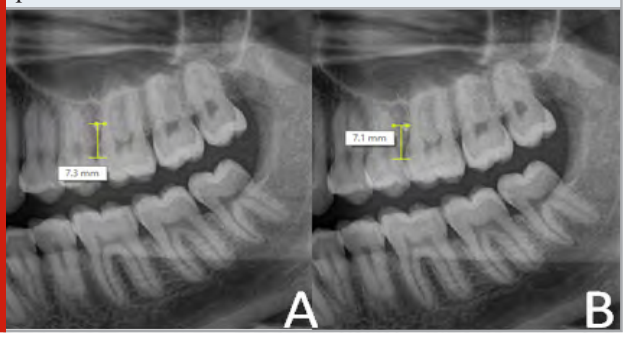


A) Point marked at 10mm distance from CEJ. B) Patient biting the putty placed on the occlusal surface, the marked point on gingiva is visible. C) The inner arm of the divider was placed on the exposed point. D) Once putty was set, the other arm of the divider closed on the cheek and a point was marked. E) Gutta percha stabilized with a transparent adhesive tape on the cheek on the point marked.

RESULTS AND DISCUSSION

The radiopaque point was located between the roots of the maxillary first molar and second premolar in the OPG of all the samples that were studied. (Figure 4). The means values of the distance of the radio opaque point (IOC_{res}) to the maxillary 2nd premolar and 1st molar were calculated. The radio opaque point (IOC_{res}) was located at a mean height of 6.8 mm from the CEJ of the 1st maxillary molar, 6.8 mm from the CEJ of the 2nd maxillary premolar (Table 1). This difference could be attributed to the angle of the radiation source to the radiopaque point on the cheek and the perpendicular distance between this point and the bone (thickness of the cheek).

Figure 3: Measurements taken on the OPG
A) Distance from CEJ of the maxillary 1st molar. B) Distance from the CEJ of the maxillary 2nd premolar.



When force is applied to a tooth, tipping or bodily movement is expected to take place. It is mainly determined by the location of the C_{res} of the tooth and the distance from the force vector to this Cr_{es}. The knowledge of the accurate location of the C_{res} which is the point of the greatest resistance to tooth movement, helps in controlling the tooth movement by different moment to force ratios. Over the years, countless methods have been employed to determine the precise location of C_{res} of teeth, (Shroff et al., 1995). A force vector needs to pass the C_{res} of the maxillary dentition and through C_{res} of the maxilla to cause pure translation (Barton, 1972) and avoid any rotational effects (Roberts-Harry, 1996)(Teuscher, 1978). For a single tooth or a group of teeth, various experimental and analytical approaches have been researched by various authors. These studies reported the C_{res} of the single tooth to be half the root length as measured from the alveolar Crest, and an apical shift of C_{res} as more teeth were incorporated into the anterior segment, (Burstone and Pryputniewicz, 1980; Kusy and Tulloch, 1986; Tanne, Hiraga and Sakuda, 1989). However, it has been known that determining the position of C_{res} of the maxilla is tedious. Some cephalometric studies have attempted to determine C_{res} of maxilla by changes in craniofacial morphology (Bench, Gugino and Hilgers, 1978).

A Clinical method to determine the C_{res} of maxilla are given by Angle and Stanley Braun in 1999 was holding an amalgam plugger in maxillary vestibule when teeth were in occlusion and soft tissues relaxed. The amalgam plugger was palpated externally to determine the C_{res} of maxilla and marked on the cheek extraorally for the outer bow to pass through. Another clinical method known is to draw a line vertically 10 mm from the outer canthus of the eye and make a horizontal from that point to meet the pupil line in front of the face which denotes the C_{res} of the maxilla. But no clinical method has been known to mark the C_{res} of the maxillary dentition.

Tanne et al concluded that C_{res} of the nasomaxillary complex to be at the posterosuperior ridge of the pterygomaxillary fissure registered on a median plane by using a finite element analysis, (Tanne, Matsubara and Sakuda, 1995). Billet et al by using a holographic method on a human macerated skull showed that C_{res} of maxilla was underneath the zygomatic process but it couldn't distinguish two different C_{res} i.e of the maxilla and maxillary dentition which are essential for application of accurate forces as suggested by Teuscher in 1986 and Stockli and Teuscher in 1994 (Billiet, 2001). Lee et al also conducted a similar study in a dry skull as Billet et al and concluded the C_{res} to be closer to the one as determined by Billet et al and couldn't investigate the C_{res} of the Upper dentition (Lee et al., 1997). All the above literature has no study to determine the C_{res} of the maxillary dentition and all the above studies are sophisticated studies.

Figure 4: represents the position of the Radiopaque point between roots of the 2nd maxillary premolar and 1st maxillary molar

Thus the objective of this study was to establish a simple, easy, quick cost effective method to locate the C_{res} of the maxillary dentition extraorally which in turn helps swift maxillary Orthopaedic appliance therapy. Thus, this study was conducted in an attempt to determine whether the centre of resistance of the maxillary dentition established previously between roots of premolars coincides with the point marked extraorally on the cheek. The results revealed that the radiopaque point marked extraorally was reproduced constantly between the roots of the 1st maxillary molar and 2nd maxillary premolars. Miki in (1979) and Hirato in (1984) reported the location of Cres of the maxillary dentition is between the first and second premolars in the anteroposterior direction and between the lower orbital margin and the distal apex of the 1st

molar vertically in the sagittal direction. (Zhang et al. 2016) This was distinguished by Teuscher in 1986, and Stockli and Teuscher in 1994 into C_{res} of the maxillary dentition to be situated between the roots of the two premolars and the C_{res} of the maxilla to be situated at the postero-superior area of the zygomaticomaxillary suture, (Teuscher 1986; Roberts-Harry 1996, Suzuki et al. 2019).

Table 1. Mean values from CEJ of maxillary 1st premolar and molar to the IOC_{res}, that is the radioopaque point on the OPG.

	DISTANCE FROM CEJ OF THE 2nd MAXILLARY PREMOLAR TO IOC_{res}	DISTANCE FROM CEJ OF THE 1st MAXILLARY MOLAR TO IOCres
TOTAL NO OF PATIENTS	7.50	6.80
	7.40	7.40
	6.80	7.30
	6.40	6.50
	7.30	7.40
	6.00	6.90
	6.10	7.10
	6.80	6.20
	7.10	7.50
	7.50	7.00
	6.40	6.10
	6.10	6.50
	6.80	6.40
	6.90	6.30
MEAN VALUE	6.81	6.79
STANDARD DEVIATION	0.483	0.528

Our results are in contradiction to the results obtained by these authors. The reasons for this transposition can be explained by the fact that OPG is a two dimensional radiographic technique. While recording an OPG, the patient remains stationary but the x-ray source rotates in front of the patient from one side to the other side. The film rotates in the opposite direction to the x-ray source behind the patient, (Pandolfo and Mazziotti, 2013). Also while recording an OPG, the different angles of radiation causes magnification of posterior teeth and due to the negative angle of the ray tube in an OPG machine this magnification is known to be higher for maxillary teeth as compared to mandibular teeth (Yassaei, Ezoddini-Ardakani and Ostovar, 2010).

This magnification might cause some distortion. The x-ray source is directed at an angle to the Gutta percha point on the cheek and not directed perpendicularly, which caused the radiopaque point on the cheek (EOCres) to appear shifted distally on the OPG and not appear exactly on the IOCres in between the roots of the maxillary premolars. The distortion can also be attributed to the distance between the soft tissue cheek on which the point was marked and the bone. But this distortion was uniformly seen in all the study samples. The vertical measurements are more reliable in an OPG as compared to horizontal measurements as in the horizontal dimension, the functional focus is the rotation centre, whereas in the vertical dimension the focus is the X-ray source. Vertical measurements are reliable which still poses the point to be in between the molar and premolar (Thailavathy et al., 2017).

Thus, we can state that, if the mean values obtained are in this range and if the point is located between the maxillary 1st molar and 2nd premolar, the point has been accurately marked and is the C_{res} of the maxillary dentition. Thus, if the point is situated at any other position other than the one determined by this study, it could be due to some error while marking the point clinically. Cephalograms were not used in this study for confirming the position of the point keeping in mind the superimposition of structures making it less reliable for landmarks (Thailavathy et al., 2017), affecting clarity and accuracy.

CONCLUSION

Thus, this novel simple method is a clinical innovation and can be used to determine the centre of resistance of the maxillary dentition extraorally for Class II patients requiring headgears, or bodily distilization of maxillary arch or maxillary arch intrusion. This method could be used by orthodontists as a chair side method which is effective and less time consuming and simple. This was a pilot study and requires a study to be conducted with a larger sample size to confirm its results and its use in orthodontic practice.

Conflict of Interest: None.

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Screening, Production and Characterization of Industrially Important Enzymes by *Serratia marcescens* Strain VT1

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ABSTRACT

Microorganisms and their enzymes are important part of industrial biotechnology because of their high adaptability, versatile metabolic machinery and simple genetic constitution which can be easily manipulated to meet different needs. A mesophilic novel strain of *Serratia marcescens* - VT1 was found to produce extracellular protease (13.173 U/ml) and cold active and stable psychrophilic lipase (23.17 U/ml). Characterization of protease showed the enzyme to be active over a wide range of pH 4-11, optimum pH 10, and optimum temperature 50°C. SMVT1 lipase was found to be active from pH 7-9 with optimum pH 7 and good stability for 90 minutes, optimum temperature 30°C and to retain almost 80 % activity at lower temperatures (20 and 10°C). SMVT 1 protease was stable in hydrophilic solvents like methanol and ethanol. Lipase showed stability in both hydrophobic and hydrophilic solvents, hexane-92.67%, methanol-87.4% and acetone-85.23%. The wide range pH active protease and psychrophilic and organic solvent stable lipase can be made use in various sectors like bioremediation, waste management, and chemical synthesis, food processing and detergent additives.

KEY WORDS: *SERRATIA MARCESCENS*, PROTEASE, LIPASE, PSYCHROPHILIC, SOLVENT STABILITY.

INTRODUCTION

The importance of microbial enzyme lies in the role played by these bio-molecules in catalyzing the broad array of reactions which is indispensable for maintaining life in earth. Enzymes can be isolated from any living forms and each one has its own characteristic property which separates it from other. In industrial sector enzymes have immense applications, among these enzymes microbial ones form an inevitable part and have always been in demand due to their unique properties (Liu and Kokare 2017). There is an increase likelihood for finding enzymes with distinctive characters in areas ranging from dry deserts to extreme cold Antarctic regions. Microbial enzymes are known to be more stable and catalyse a wide range of activities when compared to their counterparts (Hasan et al., 2006). Microbial enzymes have applications in food and beverage, diary, leather, paper and pulp, pharmaceuticals, fertilizer and detergent industries. They are also used for degradation and sustainable management of waste materials and production of bio-fuels. Among these enzymes protease remains the dominant one till to date followed by cellulase and lipase (Chapman et al., 2018 Eddehech et al. 2019 Chandra et al 2020).

Proteases (EC 3.4.21) are enzymes that increases the rate of proteolysis resulting in single amino acids, the end product of protein breakdown resulting from the cleavage of peptide bond (Barrett and McDonald, 1986). Around 60 % of enzyme that is being marketed worldwide is protease (Rao et al., 2009). About two third of the commercial protease is of microbial origin (Beg and Gupta 2003).

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Lipases (EC 3.1.1.3) are hydrolytic enzymes that generate fattyacids and glycerol by fat digestion in aqueous media. Majority of industrial lipases are microbial ones, bacterial or fungal origin (Jaeger and Reetz 1998). Cellulases (EC 3.2.1.4) are a miscellany of endocellulases, exocellulases and cellobiose that generate cellulolysis the degradation of cellulose the most abundant carbon source in earth. Majority of cellulose in earth is considered as waste, but they can be the best source of food and potential source of energy (Elder et al., 1986). Xylanases (EC 3.2.1.8) are major hemicellulases that breaks down xylan a major plant cell wall material into xylose. Xylanases and cellulases are mainly of microbial origin and have also been reported from certain marine algae, snails, crustaceans, insects and seeds of plants (Walia et al., 2013 Chandra et al 2020).

Microbial protease, lipase, cellulase and xylanase are used in textile, pulp and paper, food and beverage and animal feed industries. They are also used in the production of biofuels, and eco-friendly bioconversion and management of waste materials and in pollution control (Liu and Kokare 2017, Subramaniyan and Prema 2002, Ramnath et al., 2017, Ali et al., 2016, Romdhane et al., 2010). Proteases and lipases have importance in medicinal and pharmaceutical sector (Andualema and Gessesse 2012, Singh et al., 2016). They are also used for degumming in silk and leather industry and for synthesis of peptides and esters. Proteases are made use in recovery of silver from photographic films and X-ray, (Ali et al., 2016). The ever increasing demand for microbial enzymes is because of the specific substrate specificity, low and high temperature and pH stability and optima (Singh et al., 2016, Treichel et al., 2010). For an enzyme to be industrially useful it must be thermo stable and must maintain activity even in the presence of solvents (Hasan et al., 2006). Enzymes which are fundamentally stable and active have more use in industrial sector. The following paper deals with the screening, production and characterisation of four industrially important enzymes, protease, lipase, cellulose and xylanase by *Serratia marcescens* strain VT 1.

MATERIAL AND METHODS

Microorganism is enough: The microorganism used in the present work was *Serratia marcescens* strain VT 1 (SMVT1) was isolated from oil contaminated soil collected from Althara Devi temple in Trivandrum district of Kerala. The organism was maintained by sub culturing in olive oil enriched nutrient agar plates (peptone - 0.5 g, yeast extract – 0.5 g, NaCl - 0.5 g, agar - 2 g & olive oil 1 ml for 100 ml). The cultures were incubated at 37°C for two days and were stored at 4°C in refrigerator.

Screening for enzymes – plate assay: Protease production:Casein agar plates composed of casein - 2 g, agar- 2 g, glucose - 0.5 g, NaCl - 0.5 g, peptone - 0.5 g & yeast extract - 0.5 g in 100 ml distilled water were streaked with microbial culture and kept in incubator for 2 days at 37°C. The plates were observed for clear zones around colonies which confirm protease activity.

Lipase production: Rhodamine olive oil agar plates were used to screen the presence of extracellular lipase (Castro-Ochoa et al., 2005). The media composed of peptone - 0.5 g, yeast extract - 0.5 g, NaCl - 0.5 g, agar - 2 g, olive oil - 3 ml & rhodamine - 0.1 mg for 100ml.The inoculated plates were incubated at 37°C for 48 h and observed under UV light for fluorescent orange halos around colonies which confirms lipase production.

Cellulase production: Screening test for cellulase was done by streaking the microbial culture on to CMC (Carboxy methyl cellulose) agar plates (CMC - 0.5 g, peptone - 0.5 g, yeast extract - 0.5 g & agar - 2 g for 100 ml). The plates were incubated for 48 h at 37°C. The plates were flooded with 1 M, 0.1% Congo red for 15 minutes and washed with 1M NaCl solution. Presence of clear zone confirms cellulase activity (Gohel et al., 2014).

Xylanase production: The bacterial culture was grown on xylan containing medium (peptone - 0.06g, yeast extract - 0.06 g, MgSO4 - 0.02 g, K_2HPO_4 - 0.1 g, xylan - 0.5 g & agar - 2 g for 100 ml) for 48 h at 37°C. Congo red (1M, 0.1 %) was used for staining and 1 M NaCl was used for destaining (Subramaniyan, 2012).Clear zone around the colony confirms presence of extracellular xylanase.

Fermentation studies: Medium for protease production:The pre- inoculums was raised at room temperature for 24 h at 120 rpm in 50 ml liquid medium composed of casein - 0.5 g, glucose - 0.5 g, NaCl - 0.5 g, peptone - 0.5 g & yeast extract - 0.5 g, $CaCl_2.H_2O$ - 0.05 g, KH_2PO_4 - 0.02 g & $MgSO_4.7H_2O$ - 0.05 g. 5 ml of this culture was transferred to production media of same composition and incubated at room temperature at 120 rpm for 96 h. pH of the medium was adjusted to 7 using Na_2CO_3 and the total volume of the medium was made up to 100 ml. Samples were collected at intervals of 24 h and centrifuged at 10,000 rpm for 15 minutes at 4°C, the supernatant was stored at 4°C in refrigerator and used as crude enzyme.

Medium for lipase production: The pre- inoculums was grown in 50 ml nutrient broth (yeast extract- 0.5 %, peptone- 0.5 % & NaCl- 0.5%) by transferring a loop full of one day old microbial culture, incubated at room temperature at 120 rpm for 24 h. The production medium contains 1% olive oil in addition to pre- inoculum media, pH- 7 (Selvamohan et al., 2012). The culture conditions were exactly same. Samples were collected at a regular interval of 24 h for 4 days. Crude enzyme was obtained by centrifuging the culture to separate the cells at 10,000 rpm for 15 minutes at 4°C. The crude enzyme was stored at 4°C in refrigerator.

Protein estimation and pH determination: Cell protein was estimated according to standard procedure (Lowry et al., 1951). Bovine Serum Albumin (BSA) was used as standard. The pH values of the culture medium at different intervals were also noted.

Quantification of enzyme activity

Lipase assay: Lipolytic activity was determined by using paranitrophenyl palmitate (PNPP) as substrate (Yagiz et al., 2007). Reaction mixture (9 ml of 50 Mm Tris HCl pH - 8 containing 40 mg triton X- 100 &10 mg gum arabic mixed with 3 mg of PNPP in 1 ml propane- 2- ol) with 0.1 ml of crude enzyme was incubated at 37°C for 30 minutes, and the release of p- nitrophenol was measured calorimetrically at 410 nm. The amount of enzyme required to release 1µmol of p- nitrophenol per minute per ml from PNPP was defined as the unit enzyme activity. P- nitrophenol was used as the standard.

Protease assay: Protease activity was measured using casein as substrate (Tsuchida et al., 1986). 0.5 ml of crude enzyme was mixed with0.5 ml of substrate (2% casein in sodium phosphate buffer pH- 7) and incubated at 40°C for 10 minutes. The reaction was terminated by adding 1 ml of 10% TCA. 0.5 ml of phosphate buffer with substrate and 1 ml TCA was used as blank. The resulting mixture was centrifuged at 2000 rpm for 5 minutes. To 1 ml of supernatant 5 ml of 0.44 M Na_2CO_3 was added and incubated for 10 minutes. To this 2 fold diluted 0.5 ml Folin- Ciocalteau reagent was added and allowed to stand for 20 minutes; absorbance was measured at 660 nm. One unit of enzyme activity is defined as the amount of enzyme that released 1µmol of tyrosine per minute per ml. Bovine Serum Albumin (BSA) was used as standard.

Biochemical characterization: The biochemical characterization of crude enzyme was carried out with respect to optimum pH, pH stability, optimum temperature, temperature stability and effect of solvents.

Optimum pH and pH stability: Optimum pH for enzyme activity was determined at different pH values ranging from 4-11 by preparing substrates in appropriate buffers (protease: 4 & 5- sodium acetate buffer, 6 to 8- sodium phosphate buffer and 9 to 11- sodium carbonate buffer, lipase: 4 & 5- sodium acetate buffer, 6- sodium phosphate buffer, 7 to 9- Tris HCl and 10 & 11- sodium carbonate buffer). Enzyme assays were done according to the above mentioned procedures. The effect of pH on enzyme stability was studied by incubating the enzyme in desired buffer with optimum pH for two hours and calculating the residual activity for every half an hour.

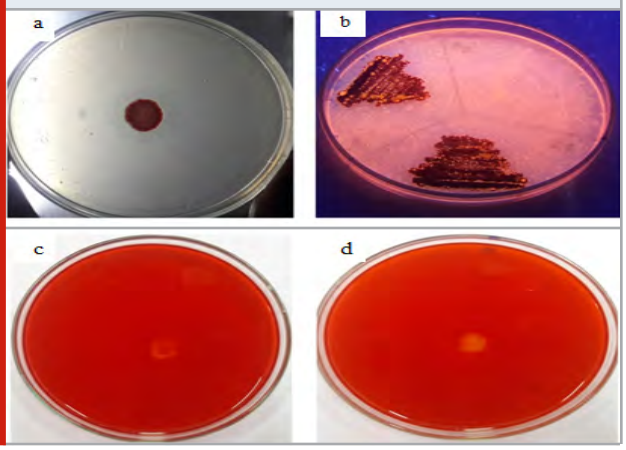
Optimum temperature and temperature stability: The optimum temperature was determined by performing respective enzyme assay at different temperatures ranging from 30- 80°C. Thermal stability was estimated by pre-incubating the crude enzyme at optimum temperature for two hours. Residual activity was determined for every half an hour.

Effect of solvents on enzyme activity: The different solvents used in the study ethanol, methanol, butanol, isopropanol, chloroform, ethyl acetate, acetone and hexane were selected based on their log P values. Crude enzymes were mixed with solvents to make a final concentration of 20% (v/v); the mixture was mixed well and stored at 4°C. The residual activity was determined after two hours of incubation by colorimetric method. Enzymes mixed with distilled water 20% (v/v) was used as control.

RESULTS AND DISCUSSION

S. marcescens VT 1 was subjected to screening by plate assays to confirm the presence of extracellular proteases, lipases, cellulases and xylanases (Fig: 1). After 48 h of incubation clear zone was visible around the bacterial colony in casein agar plates confirming protease activity. Hydrolysis of casein and gelatin was used as the preliminary test for isolation of protease producing *Serratia* sp RSPB11 (Bhargavi and Prakasham 2012). 14 protease producing strains were isolated on casein agar plates (Vakilwala and Patel 2017). Under UV light orange fluorescent halos were clearly visible resulting from the reaction of fatty acids and rhodamine pointing towards expression of extracellular lipase. Extracellular lipase production of *Serratia marcescens* and *S. aureus*, on Rhodamine B plates have been previously reported (Kouker and Jaeger 1987). Ejike and coworkers (2017) used Rhodamine B plates for preliminary screening of lipase producing organism *S. marcescens*. Majority of the lipases produced by microorganisms are extracellular inducible enzymes transmitted to outer surface (Ota et al., 1982). Clear zones were completely absent in CMC agar plates and xylan agar plates, so it can be inferred that cellulase and xylanase production is absent, therefore as the next part of work the production pattern and biochemical characterization of protease and lipase were studied.

Figure 1: Plate Assay (A) Protease, (B) Lipase, (C) Cellulase And (D) Xylanase From SMVT1



The enzyme production pattern was evaluated along with the change in pH profile of the medium and concentration of cell protein for a period of 96 h (Fig: 4 and 5). The extracellular protease production started with the log phase of growth and activity reached the peak value after 24 h (13.173 U/ml), thereafter the activity declined with time and showed a slight increase at 96 h (Fig: 2). The lipolytic activity of *S. marcescens* VT 1 was measured by colorimetry using PNPP as substrate at 420

nm. Study showed that the lipase activity was expressed maximum after 24 h of incubation, further a turn down in activity is seen which is followed by a rise in activity at 96 h (Fig: 3). The maximum activity recorded was 23.17 U/ml followed by 20.16 U/ml.

Figure 2: Enzyme Activity- SMVT1 Protease

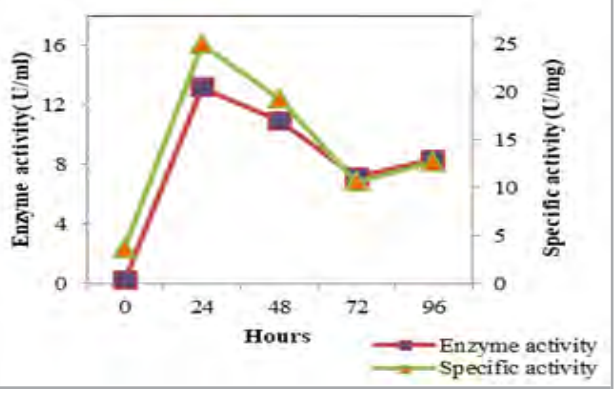
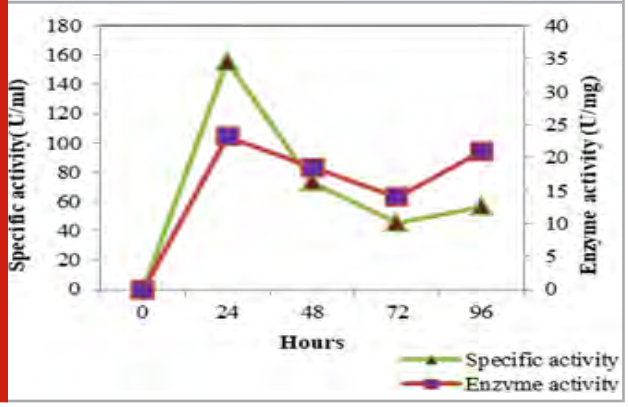


Figure 3: Enzyme Activity- SMVT1 Lipase



S. marcescens protease has been studied and described in previous papers (Henriette et al., 1993, Romero et. al., 2001, Ustariz et. al., 2008). Excretion of extracellular protease of *S. marcescens* ATCC 25419 was reported to occur during the logarithmic growth phase and was determined to be highest during the stationary growth phase et al., (Schmitz and Braun 1980). Lipid hydrolysis by *S. marcescens* was estimated and recorded earlier (Heller 1979, Prasad 2013, Abdou 2003). Henriette et al., (1993) based on their study reported that the *S. marcescens* released lipase during the stationary phase of growth after growing exponentially at 22°C for 6 h. Lipase production was found to begin after 5- 10 hours of growth at 27°C (Makhzoum et al., 1995). Proteolytic activity along with lipolytic activity of *S. marcescens* has been previously studied and recorded (Henriette et al., 1993, Begam et al., 2012, Abdou and Ohashi 1996). The rise in activity at 96 h may be due to the release of intracellular lipase into the medium due to death and lyses of bacterial cells. The pH of the protease medium was found to shift towards the alkaline side with time and growth of *S. marcescens* VT 1, while the pH of lipase medium turned alkaline first and later the alkalinity started to drop off after 48 h. The release of fatty acids, the byproduct of lipolysis into the medium could have resulted in reduction of alkalinity.

Figure 4: Cell Protein Profile Variation During Protease And Lipase Production By SMVT1.

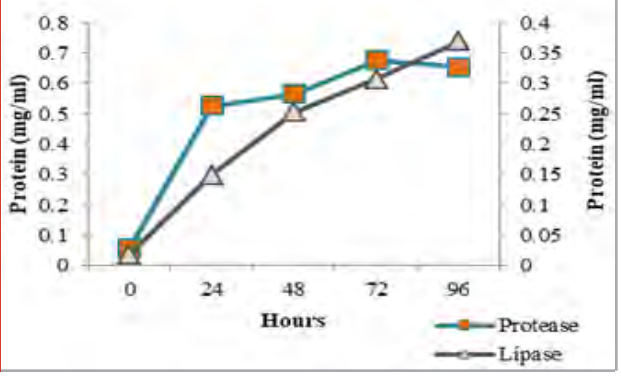


Figure 5: Variation In pH Values During SMVT1 Protease And Lipase Production.

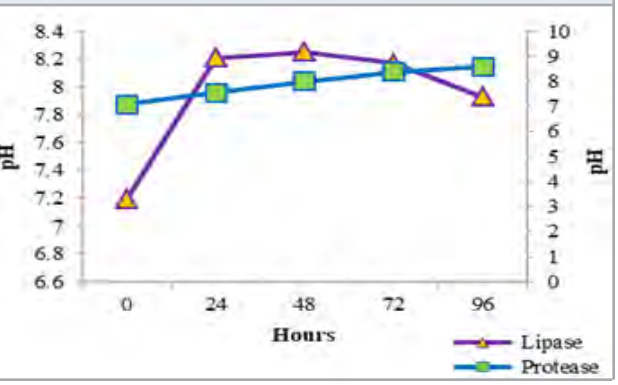


Figure 6: Optimum pH Of Protease And Lipase

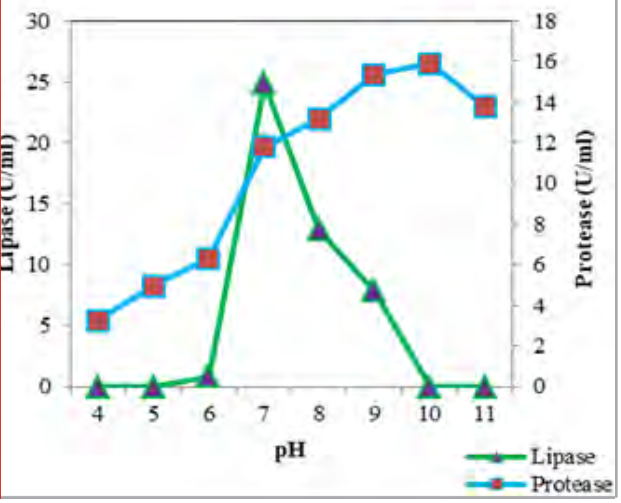
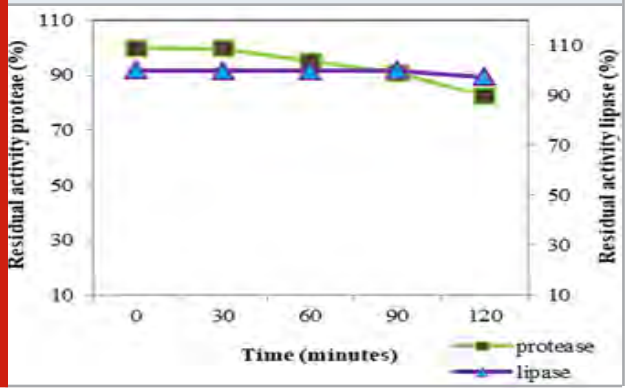
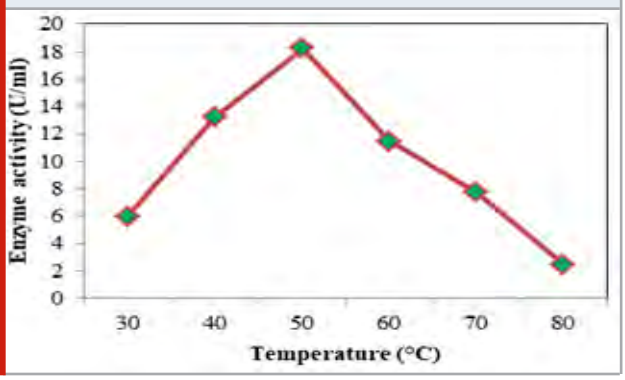


Figure 7: Ph Stability o Protease and Lipase



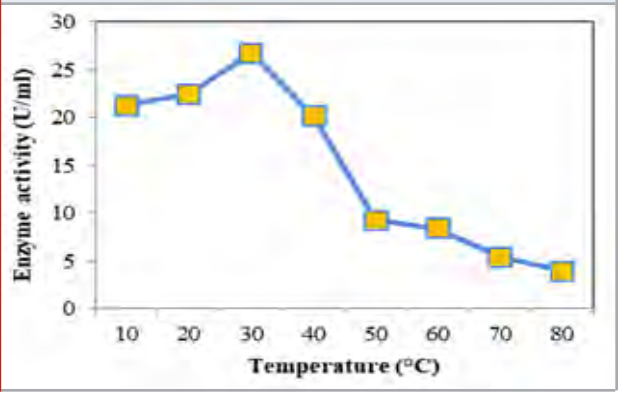
The optimum pH (Fig: 6) for protease was found to be 10 (15.887 U/ml). At pH 9 the activity was found to be almost similar (15.346 U/ml). The proteolytic activity was found to decrease as the pH shifted towards acidic range. From this it is clear that the lipase of *S. marcescens* VT 1 is an alkaline protease and is active over a wide range of pH. Annapurna and colleagues (Annapurna et al., 2012) reported an alkaline protease form *S. marcescens* with optimum pH 10. *S. marcescens* metalloprotease SMP 6.1 with pH optimum of 10 was also reported (Salamone and Wodzinski 1997). Protease with an optimum pH of 8 and pH range of 6 to 11 was recorded in *S. marcescens* (Femi-Ola et al., 2014). Kim et al., (2007) reported the optimal pH of protease by *S. marcescens* as 7 using casein substrate. *S. marcescens* TKU019 protease exhibited a broad pH range 5-10 (Liang et al., 2010). The optimum activity of lipase was estimated at neutral pH. A sharp decline in activity was observed when pH shifted to 6 and activity was found to be absent at pH 5 and 4. Activity was found to decrease as the pH was raised and was lost at pH 10 and 11. *S. marcescens* lipase optimum pH was found to be 6.5 (Gao et al., 2004), 8 and retained 95% of maximum activity at 7 (Zaki and Saeed 2012) and 8-9 (Abdou 2003).

Figure 8: Optimum Temperature Of SMVT1 Protease



The pH stability (Fig: 7) of VT 1 protease was studied by incubating the crude enzyme in pH 10 for two hour at 4°C; assay was conducted every half an hour. The enzyme was found to be stable for 30 minutes in pH 10, after 60 minutes the enzyme retained 96.15% activity. After 2 h the enzyme lost 17.63% of its original activity. *S. marcescens* protease was found to be stable over a pH range of 5 to 10 under low temperature incubation and underwent inactivation in alkaline pH on incubation under elevated temperature (Miyata et al., 1970). Purified *S. marcescens* protease remained stable for 1 h at pH ranging from 6-9 and lost 40% of stability at pH 10 (Iqbal et al., 2018). For lipase pH stability study was conducted by incubating enzyme in Tris HCl buffer pH 7 and estimating activity every 30 minutes for two hours. VT 1 extracellular lipase was found to be stable for one and half hours and lost 2.73% of the original activity in the next 30 minutes and retained 97.27% activity by the end of two hours. *S. marcescens* lipase showed good stability between pH 6-9 (Makhzoum et al., 1995).

Figure 9: Optimum Temperature of SMVT1 Lipase



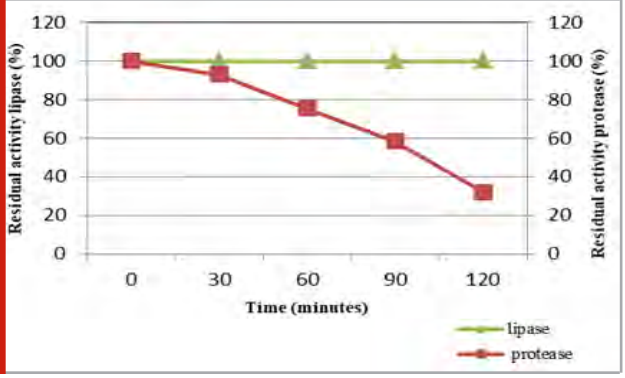
Lipase from *S. grimisii* remained stable over the pH range 7-9 et al., (Abdou 2003). Abdou et al.,(2003) found that purified *S. marcescens* lipase showed maximum stability at pH 8. In a study by Zaki and Saeed (2012) purified lipase from *S. marcescens* showed maximum stability at pH 8.

Optimum temperature for protease was assayed at temperature from 30 to 80°C; casein was used as the substrate at pH 7. Optimum activity recorded was 18.232 U/ml seen at 50°C (Fig: 8). At higher and lower temperatures the activity was found to decrease. For alkaline proteases the optimum temperature falls in the range 50- 70°C (Kumar and Takagi 1999). Femi-Ola and fellow workers (2014) obtained optimum proteolytic activity for *S. marcescens* at 50°C, at temperatures above and below reduction in activity was observed. Optimum activity was reported at 67°C for *S. marcescens*, alkaline protease (Annapurna et al., 2012). The optimum temperature of *S. marcescens* protease was found to be 50°C (Liang et al., 2010) and 42°C (Salamone and Wodzinski 1997). Optimum temperature for lipase (Fig: 9) was initially evaluated at temperature range 30- 80°C. Since the optimum activity was at 30°C (26.736 U/ml), lower temperatures 10 & 20°C were also considered. Optimum temperature for lipase from *S. marcescens* was determined as 35°C (Zaki and Saeed 2012).

S. marcescens lipase optimum temperature was reported to be 37°C (Abdou 2003), between 25- 35°C (Immanuel et al., 2008). With increase in temperature the lipolytic activity was found to decrease showing a sharp decline from 40- 50°C. At lower temperatures (30 to 10°C) the enzyme activity showed a slight decrease only, the activity was found to decrease by only 5.4 U/ml retaining almost 80% of the maximum activity. This shows the psychrophilic property of lipase. Abdou (2003) observed high lipase activity for *S. marcescens* at 5°C and low temperature activity of *S. grimisii*. Cold active enzymes have much economic and ecological benefit when compared with their equivalents which need high temperature to function (Marchi et al., 2007) Psychrophilic lipases have high catalytic activity and consume less energy at low temperatures which makes them efficient tools in the production of detergent, leather, food, pharmaceuticals, fine chemical and bioremediation (Joseph et al., 2008).

Temperature stability of VT 1 protease and lipase (Fig: 11) were studied at 50°C and 30°C (optimum temperature) respectively for a period of 2h and residual activity was determined after every half an hour. The VT 1 protease was active throughout the study period of two hours, but the activity was found to decrease drastically after 30 minutes. After 30 minutes of incubation at 50°C the activity decreased by 7%, thereafter the activity was found to lower by 61%. The lipase of *S. marcescens* was stable up to 45°C and lost 75% of stability at 60°C after 1 h of incubation (Iqbalet al., 2018). Matsumoto and partners (Matsumoto et al., 1984) showed the complete inactivation of *S. marcescens* protease above 65°C. Miyata et al., (1970) displayed the protease fro *Serratia* spp. to be relatively stable at 40°C throughout the 60 min of study, but mere heating for 15 min at 50°C resulted in complete inactivation.

Figure 10: Temperature Stability of SMVT1 Protease And Lipase.



The lipase of VT 1 was thermo stable throughout two hours, showing that the lipase is highly stable at lower temperature. The *S. marcescens* lipase showed least residual activity at 85°C and was completely inactivated at 90°C. *S. marcescens* lipase was exhibited to be less thermostable compared to other psychrotroph lipases, at the same time is resistant to inactivation at lower temperatures (Abdou 2003). Thermal stability of *S. marcescens* lipase in the culture was showed to decrease with increasing temperatures (Gao et.al., 2004. After 24 h the residual activity was found to decrease by 61% and 36% after incubation at 35°C and 25°C.

Table 1. Solvent stability of SMVT1 protease and lipase

Solvents	log P	Residual protease activity (%)	Residual activity (%) lipase
Methanol	-0.69	79.71	87.4
Acetone	-0.61	54.73	85.23
Ethanol	-0.18	82.28	76.55
Isopropanol	0.16	20.06	28.09
Ethyl acetate	0.71	38.83	37.63
Butanol	0.839	59.34	34.73
Chloroform	1.67	33.83	71.39
Hexane	3.769	65.79	92.67

Solvent stability of the enzymes were studied in eight different solvents with different log P values, which represents hydrophobicity level (Table: 1). Lower the log P value lower the hydrophobicity. Protease was found to be stable in polar solvents like methanol and ethanol, unlike the previously reported solvent tolerant protease from *S. marcescens* MH6 which displayed notable stability in hydrophobic solvents (Wan et al., 2010). Protease showed some stability in nonpolar solvent hexane also. Least amount of residual activity was observed in isopropanol and chloroform. *Serratia* sp. SYBC H protease retained over 90% activity even after 60 minutes of incubation at 40°C in 50% (v/v) hydrophilic organic solvents such as dimethylformamide, dimethylsulfoxide, and acetone (Li et al., 2010). The *S. marcescens* PPB-26 protease showed maximum stability in methanol and ethanol and was stable in all organic solvents except isopropanol (Thakur et al., 2016).

The VT 1 lipase showed higher stability in methanol, acetone, and ethanol which are polar solvents with lower log P values, however maximum stability was observed in a nonpolar solvent hexane retaining 92% of activity after 2 h of incubation. Lipases displaying stability in a wide group of organic solvents, regardless of their log P values have industrial importance in esterification, interesterification, and transesterification alias synthesis (Chakravorty et al., 2012). *Serratia marcescens* ECU1010 lipase presented commendable stability in many water miscible and immiscible solvents (Zhaoet al., 2008). Lipase from *Pseudomonas reinekei* displayed significant stability in hydrophilic solvents like ethanol and methanol (20% v/v) after 24 h of incubation (Priyanka et al., 2019). The activation of lipase in hydrophilic and hydrophobic solvents can be explained due to the amino acid solvent interactions leading to opening of the lid or flap covering the catalytic site keeping the enzyme

in an open conformation (Bose and Keharia 2013, Cao et al., 2012).

CONCLUSION

S. marcescens strain VT 1 a soil bacterium was isolated for the production of industrially important enzymes and was found to produce lipase and protease. A further characterization study showed the protease to have an optimum pH of 10 and was active over a wide pH range of 4-11 and showed stability at pH 10. Lipase had an optimum pH of 7 and was active in a pH range of 7-9 with good stability at pH 7 for 90 minutes. The optimum temperature of protease and lipase was found to be 50 and 30°C. Lipase was found to be cold active and incredibly stable at low temperatures. SMVT 1 protease was found to be stable in hydrophilic solvents like ethanol and methanol, while lipase showed stability in solvents with both low and high log P values. *S. marcescens* strain VT 1 protease and lipase can be of great importance in industries like food processing, biodiesel production, and waste management.

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On The Analysis of Certain Biochemical Parameters of Carps Cultured in Domestic Sewage Oxidation Ponds

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ABSTRACT

Reclamation of waste water for protein production is a concept that is being paid more attention of late, as it is an environmental friendly, economic and experimental interdisciplinary process to produce large quantities of edible fish. Domestic sewage is full of minerals, nutrients and organic matter which suits for aquaculture production without any supplementary feeding and it is also an economically viable biological treatment of domestic waste waters. In the present study changes in tissue glycogen and serum glucose contents of three fishes belonging to the carp family, cultured in secondary domestic waste oxidation ponds have been analysed. Tissue glycogen values in liver, kidney and muscle and serum glucose of *C.carpio, L.rohita* and *C.mrigala* cultivated in secondary sewage ponds were estimated. Tissue glycogen was found highest in liver, followed by muscle and kidney, in *C.carpio* grown in seconadary ponds of domestic sewage, as compared to the other fishes. Serum glucose value was also found highest in *C.carpio* which is a voracious polyphagous feeder. The results of our findings support the fact that there is improved fish growth in sewage ponds which are full of nutrients and minerals, enabling a symbiotic process of reclamation of proteins as well as biological treatment of the sewage. More bio-chemical assessment of fishes grown in sewage ponds are needed to remove the mis concept that sewage cultured fishes are not safe for human consumption

KEY WORDS: SEWAGE, CARP CULTURE TISSUE GLYCOGEN LIVER, KIDNEY MUSCLE.

INTRODUCTION

Water is one of the vital resources for all the lifeforms and it is also the resource which is most adversely affected by human activities, especially after rapid growth in industrialization and urbanization. It is known that the aquatic eco-systems are most delicately balanced and get easily disrupted by various human activities (Chiou et al. 2007). Of various types of human activities, sewage disposal continues to be the most ominous one. Sewage coming from living human quarters consist of biodegradable organic matter and faecal matter. Before disposing into fresh waters, sewage water has to be treated which gave rise to the concept of oxidation or stabilization ponds (Bhatia et al 1970, Hepher and Schroeder (1977) Wu et al. 2013; Kumar et al. 2015). These oxidation ponds are eco-friendly environmental & economical technique to grow fish and prawn (Siddig et al. 2019). Fishes grown in sewage waters exhibit faster growth as sewage is composed of high nutrients, organic and protein contents. It is also observed that these fishes are not harmful for human consumption (Mannacharaju et al. 2020).

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Water reuse or reclamation of waste water for aquaculture from treated municipal effluent waters is a useful technique which is being practiced worldwide. The first recorded scientifically carried out experiment of culturing fish in sewage ponds was done by Mayenne (1933) in Germany. Later on countries like Japan, Taiwan, Indonesia, Phillippins, Hongkong, Malaysia etc., started sewage pond culture of fishes. In India, fish culture practices in fresh water ponds fertilized with domestic sewage started in 1940's, especially in West Bengal, Tamilnadu and other few states. (Modak 1938; Vaas, 1940; Hora 1944; Pillai et at. 1945; Ganapati & Chacko 1950; NEERI, 1985; Saha et al. 1958; Bhatia et al. 1970; Allen et al. 1979; Hepher & Schroeder, 1977; Allen and Carpenter 1977; Jhingran, 1984; Ali 1992; Tripati, 1992; Bunting et al. 2001; Nandeesha, 2002; Datta, 2007; Drechsel et al. 2009; Shakir et al. 2014; Darko and Azanu, 2016; Das et al. 2020).

To find out the physiological and growth aspects of fishes grown in domestic sewage fed ponds, biochemical parameters were studied in the present study. Teleost fishes like *Cyprinus carpio, Labeo rohita* and *Cirrhinus mrigala* were grown in secondary sewage ponds and their tissue glycogen in liver, kidney and muscles and serum glucose were analysed regularly in 3rd month, 6th month, 9th month and 12th month of a year. The data was compared with the data of the biochemical parameters obtained from the same fishes grown in fresh water ponds fed with supplementary feed.

MATERIAL AND METHODS

Study area: The experimental work was made in Shahpura sewage oxidation ponds located at T.T. Nagar, 10 km south-east of Bhopal city (250-17'). There are 8 sewage oxidation ponds constructed in two series of primary and secondary as per specifications of National Environmental Engineering Research Institute (NEERI), Nagpur. Each pond is having an area of 0.4 hectares. The ponds were typical sewage oxidation or stabilization ponds, designated to treat biologically 3 million gallons of domestic sewage per day. Sewage from adjacent areas was collected in a sump near Habibganj Railway Station, from where it was pumped to the oxidation ponds and it was detained for a period of about 15 to 20 days for microbiological transformation. The raw sewage enters the primary pond through 3 inlets and after the detention period the biologically treated effluent goes out from secondary pond through the outlet.

In the present study out of 8 ponds, as mentioned above, four were selected for fish culture. Two being primary, designated as IA & IIIA and two as secondary called as IB & IIIB. Fish seed of *C.carpio, L.rohita* & *C.mrigala* is stocked in all the above ponds but due to high concentration, no fish survived in primary ponds. The fishes grown in secondary ponds (IB & III B) were collected and the biochemical experiments were conducted as per standard procedures. The netting of fishes in secondary sewage ponds and control fresh water pond at regular 3-4 monthly intervals has been done using standard nets. The experiment was conducted for a period of one year.

Estimation of Biochemical Components: Live, mature and healthy *C. carpio, L.rohita* and *C.mrigale* were caught from sewage oxidation ponds and immediately brought for experimentation. After 1-2 hrs of laboratory acclimatization, the fishes were used for blood analysis. By severing caudal peduncle artery blood was collected in the pre oven sterilized centrifuge tubes and subjected for centrifugation for a period of 5 minutes at 3000 rpm. The centrifuge tubes were stored at 40 ^{0}C for serum precipitation.

The caudal peduncle severed fishes were dissected and liver, kidney and muscle were exposed and kept in cold storage for analysis of biochemical parameters. A 5% tissue homogenate was prepared using Potter-Elvehjem homogenizer at 10000 rpm for 10-15 minutes under ice cooled conditions used for biochemical studies. Six species were used separately for each experiment. All the experiments were repeated for 3-4 times and the data were statistically analysed using standard methods (Fischer, 1950; Lewis and Lewis, 1971). Tissue Glycogen: Tissue glycogen was estimated by the glucose oxidase method described by Plummer (1978). All the chemical and reagents used were obtained from Sigma (USA). Tissue glycogen values were expressed in µ gm/g wet weight of the tissue. All the values are mean of 3-4 replicates.Serum glucose: Serum glucose was estimated according to the methods of and Folin and Wu (1929) as cited in Hawk's Physiological Chemistry, Edited by Bernard L. Oser (1965). Serum glucose values were expressed as µg per 1 ml of serum.

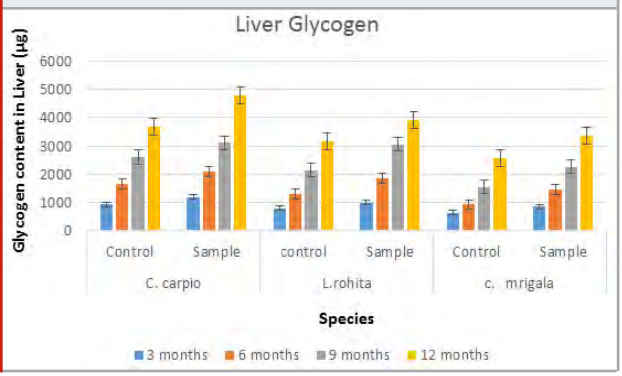
RESULTS AND DISCUSSION

The analysis of tissue glycogen was done from liver, kidney and muscle of the domestic sewage and control pond cultured fishes. It was observed that liver recorded highest content of glycogen followed by muscle and kidney from fishes grown in secondary oxidation ponds. Glycogen content in the liver of *C.carpio*, cultured in oxidation ponds for a period of 3 months exhibited significant greater values from that of *C. carpio* grown in the fresh water control pond for the same period. It was observed that the 3 months *C. carpio* from oxidation pond had 1201.16 µg/glycogen in the liver whereas the liver glycogen content in the fresh water control fish was 932.91 µg only. On the other hand 3 months old *L.rohita* cultured in oxidation ponds along with *C.carpio* showed significantly less glycogen content in

the liver than *C.carpio* and it was found to be 1000.99 µg. Similarly, the fresh water cultured *L.rohita* exhibited 789.46 µg of liver glycogen. Minimum glycogen content was observed in both the experimental and fresh water control *C. mrigala* during the 3 months old stages among the three fishes. The value was 853.13 µg in the fish from the oxidation ponds and 638.98 µg in the fish from the control pond.

During 9 months stage, *C.carpio, L.rohita* and *C.mrigala* cultured in oxidation ponds showed liver glycogen values as 3108.74 µg, 3047.11 µg and 2265.54 µg respectively. On the other hand the same fishes from the fresh water contol pond showed comparatively less liver glycogen values being 2601.79 µg, 2149.30 µg and 1543.67 µg respectively.After 12 months of culture in oxidation ponds, *C.carpio* of oxidation ponds showed maximum glycogen content in its liver i.e., 4791.78 µg whereas the same fish cultured in fresh water showed 36789.60 µg of liver glycogen content. The other fishes *L.rohita* and *C.mrigala* from oxidation ponds exhibited 3903.55 µg and 3362.30 µg of glycogen values in their liver. The same fishes *L.rohita* and *C.mrigala* cultured in control pond showed 3157.84 µg and 2569.81 µg of liver glycogen (Figure 1).

Figure 1: Showing liver and glycogen content of fishes cultured in secondary oxidation ponds for a year along with fishes cultured in fresh water as control

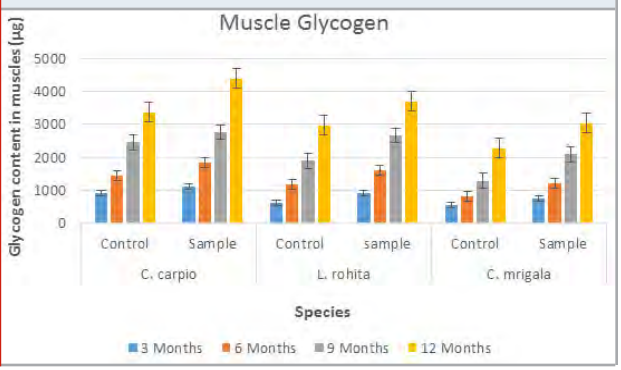


It was observed that *C.carpio* among the three fishes exhibited highest content of glycogen in its liver followed by muscle, *C.carpio* cultured from the oxidation ponds showed 1103.64 µg of glycogen in the muscle during the 3 months stage. On the other hand the fresh water control fish showed 908.28 µg of muscle glycogen content at the same age of 3 months. Similarly, the other fishes *L.rohita* and *C.mrigala* grown in sewage ponds exhibited 913.66 µg and 738.16 µg muscle glycogen whereas the *L.rohita* and *C. mrigala* cultured in fresh water pond showed 616.21 µg and 564.62 µg of muscle glycogen respectively. *C.carpio* cultured in oxidation ponds showed 1843.75 µg of glycogen in the muscle at 6 months stage whereas the 6 month control pond fish showed 1438.31 µg of glycogen in the muscle. Similarly, *L. rohita* grown in secondary sewage ponds during 6 months exhibited 1624.36 µg muscle glycogen and its fresh water control pond counterpart recorded 1182.48 µg glycogen in its muscle. It was found that *C.mrigala* cultured in oxidation pond exhibited 1208.76 µg of muscle glycogen at the stage of 6 month and its control counterpart showed 813.48 µg of muscle glycogen.

During the analysis of 9 month oxidation pond-cultured *C.carpio*, it was observed that 2763.60 µg of glycogen were present in the muscles whereas freshwater grown fish exhibited 2458.11 µg of glycogen in its muscle at the same age of 9 months. Similarly, the other fishes *L.rohita* and *C.mrigala* grown in secondary sewage ponds recorded 2664.87 µg and 2092.50 µg of muscle glycogen during 9 month stage. The fresh water pond cultured *L.rohita* and *C.mrigala* at this stage of 9 months exhibited 1898.74 µg and 1292.16 µg of glycogen in their muscles which is significantly less than the fishes grown in oxidation ponds.

At the stage of 12 months of culture of fishes, it was observed that all the three fishes recorded high amount of muscle glycogen, next to that of liver glycogen. The fishes *C.carpio, L.rohita* and *C.mrigala* cultured in oxidation ponds at the stage of 12 months exhibited 4388.43, 3696.72 µg and 3041.38 µg of muscle glycogen respectively. On the other hand the same fishes *C.carpio, L.rohita* and *C.mrigala* from fresh water pond recorded 3372.57 µg, 2968.36 µg and 2278.64 µg of glycogen content in the muscles respectively during the 12 months of culture (Figure 2).Thus it is evident that the fishes *C. carpio, L.rohita* and *C.mrigala* cultured in sewage oxidation ponds recorded high amounts of liver and muscle glycogen from the initial stages as the sewage ponds are highly nutrient. The normally fed fresh water control pond cultured fishes recorded less glycogen values (Figure 2).

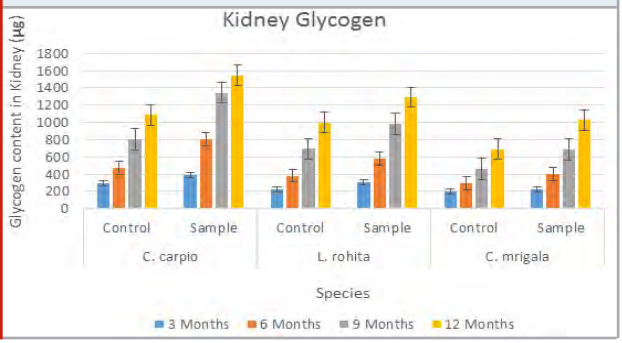
Figure 2: Showing muscle glycogen content of fishes cultured in secondary oxidation ponds for a year along with well-matched controls



During the analysis of glycogen from kidneys it was found that all the three fishes *C. carpio, L. rohita* and *C. mrigala* showed minimum glycogen content in their

kidneys. Data from 3 month old C.carpio cultured in oxidation ponds exhibited 388.68 µg of glycogen in their kidneys whereas the same 3 month old fresh water control cultured fish showed 294.28 µg glycogen in its kidney. Similarly, the other fishes *L.rohita* and *C. mrigala* cultured in oxidation ponds for 3 months showed kidney glycogen of 301.62 µg and 228.58 µg respectively. On the other hand, *L. rohita* and *C. mrigala* cultured in fresh water pond showed 218.81 µg and 196.63 µg of glycogen in their kidneys (Figure 3).

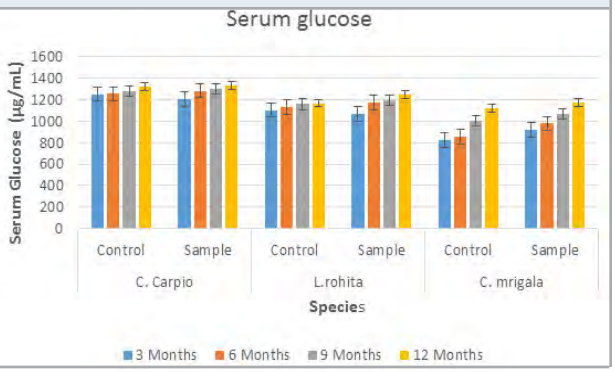
Figure 3: Showing kidney glycogen content of fishes cultured in secondary oxidation ponds for a year along with control fishes



In the next analysis i.e. at 6 months old stage, *C. carpio* from oxidation pond showed 806.71 µg of glycogen in its kidney and the same 6 months old *C. carpio* culture din fresh water showed only of 472.64 µg of kidney glycogen. The other fishes *L. rohita* and *C.mrigala* cultured in oxidation ponds exhibited 580.10 µg and 401.16 µg of glycogen in their kidneys.During 9 months of culture of fishes in oxidation ponds, *C.carpio, L.rohita* and *C.mrigala* exhibited 1345.42 µg, 981.20 µg and 688.30 µg of glycogen contents in their kidneys respectively. On the other hand, the fresh water control fishes *C.carpio, L. rohita* and *C.mrigala* after 9 months of growth exhibited kidney glycogen values of 802.46 µg, 692.66 µg and 461.84 µg respectively. At the end of one year, the analysis of kidney revealed that the experimental *C.carpio* exhibited 1542.93 µg of glycogen in its kidney. On the other hand, the control fresh water 12 months old *C. carpio* recorded 1087.50 µg of kidney glycogen. The other fishes *L.rohita* and *C. mrigala* from oxidation ponds after 12 months of culture exhibited 1292.61 µg and 1026.98 µg of glycogen content in their kidneys and the fresh water *L.rohita* and *C.mrigala* recorded 998.44 µg and 689.10 µg of kidney glycogen (Figure 3).

The analysis of serum glucose of the secondary oxidation and fresh water species of *C.carpio, L.rohita* and *C.mrigala* was also carried out. Except at the initial stage of growth i.e., at 3 months, maximum glucose values were recorded in C.carpio followed by *L.rohita* and *C.mrigala* cultured in oxidation ponds. At the stage of 3 month, *C.carpio* from oxidation pond exhibited 1200.37 µg/ml of serum glucose whereas the control fresh water *C.carpio* exhibited 1248.91 µg/ml of serum glucose during 3 months stage. Similarly, the other fishes *L.rohita* and *C.mrigala* cultured in oxidation ponds, along with *C.carpio* showed serum glucose values of 1062.46 and 918.50 µg/ml during three months culture. On the other hand, 3 month old fresh water cultured *L.rohita* and *C.mrigala* showed 1100.55 and 824.41 µg/ml of serum glucose values. During the next analysis of fishes at 6 months age, high glucose contents were found in all the fishes, cultured in oxidation ponds. *C.carpio* grown in oxidation ponds for 6 months, showed 1280.21 µg/ml of serum glucose whereas *C.carpio* during 6 months growth in fresh water pond showed serum glucose of 1250.19 µg/ml. Similarly, the other fishes *L.rohita* and *C.mrigala* from oxidation ponds during 6 month stage exhibited 1170.83 and 975.25 µg/ml of glucose whereas the 6 months old control fresh water *L.rohita* and *C.mrigala* showed serum glucose of 1128.20 and 855.39 µg/ml respectively.

Figure 4: Showing serum glucose level in fishes cultured in secondary oxidation ponds



C. carpio, L.rohita and *C.mrigala* grown in sewage ponds for 9 months showed serum glucose of 1298.64, 1192,51 and 1061.57 µg/ml respectively. On the other hand, *C.carpio. L.rohita* and *C.mrigala* from fresh water control pond exhibited 1279.15, 1154.10 and 1005.36 µg/ml of glucose respectively.Analysis of fishes after 12 months for serum glucose found that maximum glucose values were recorded in *C.carpio* followed by *L.rohita* and *C.mrigala*. *C.carpio* grown in oxidation ponds during 12 months stage showed 1330.66 µg/ml of glucose content whereas its 12 month old fresh water control counterpart showed glucose content of 1318.80 µg/ml. Similarly the other fishes *L.rohita* and *C.mrigala* from secondary oxidation ponds during one year stage exhibited 1240.53 and 1170.10 µg/ml of glucose content. On the other hand 12 month old fresh water *L.rohita* and *C.mrigala* showed glucose content of 1164.35 and 1120.50 µg/ml. (Figure 4)

Thus throughout the study period high serum glucose values were recorded in *C.carpio* compared to other fishes like *L.rohita* and *C.mrigala* cultured in domestic secondary sewage ponds.

As compared to other two species studied in the present study, *C.carpio* showed highest glycogen levels due to different dietary habits, amount of food consumed, metabolic activities and with high to that environment and physiological tolerance. As *C.carpio* is a polyphagous and a carbohydrate feeder hence it is more active than other fishes. Thus, higher levels of tissue glycogen as detailed in the carps cultured in domestic waste waters is in agreement with the concept that easy availability of nutrient rich food chain enhances the carbohydrate lipid and protein contents of fishes. Liver glycogen represents as the central reserve of fuel for the body tissues and also maintains the level of blood glucose in higher animals including fishes. It is also known that in well-fed animals, glucose is converted to glycogen in the liver and the blood glucose in tissues is maintained by the synthesis of glucose from non-carbohydrate sources i.e., glucogenesis (Plummer, 1978). It has been revealed that a significant relationship exists between dietary state and liver glycogen content (Plummer, 1978; Furukawa et al. 2018).

The reason for higher glycogen levels in liver and muscle may be due to a greater food intake and enhanced glycolytic metabolic pathways found in the experimental carps i.e., fishes grown in domestic sewage oxidation ponds. As very large quantity of nutrient rich food was consumed by the fishes in sewage waste waters, their growth pattern was quite different than that of fresh water fishes which were rather poorly fed, as they were only given supplementary feeding as per standard applications (Jhingran, 1984). The experimental fishes grew 2-3 times faster in growth than the control fishes because of higher food conversion efficiencies associated with higher enhanced metabolic rates. The carps cultured in domestic sewage secondary oxidation ponds utilizes the complete food columns of the eutrophic water and grew fast. There is no doubt that the domestic sewage influent had several important nutrients being many times rich in carbohydrates, fats, proteins and other nutrient stimulators (Mohapatra et al. 2012).

Another reason for higher content of glycogen in liver of all carp fishes was due to the fact that liver being a red tissue is instrumental in all metabolic activities and red muscles also contain hemoglobin, triglycerides, blood capillaries and their capacity to oxidize long chains of saturated fatty acids and acetate is higher than that of white muscles (Shaffi et al. 1977; Chang et al., 2020). Thus the data of the present study that higher content of glycogen was present in the order of liver followed muscle and kidney are in full corroboration with the findings of Plummer (1978); Swaleh et al. (2019); Chang et al. (2020).

In the present investigation serum glucose levels were also estimated for the carps *C.carpio, L.rohita* and *C.mrigala* grown in domestic secondary sewage oxidation ponds and compared with the control ones. The reason for high serum glucose levels of fishes grown in domestic secondary sewage waste waters may be due to their increased feeding habits and their active physiological activities. It is well known that the blood glucose levels in fishes are significantly related with their dietary habits, feeding and metabolic efficiencies. Active swimmers have high glucose levels as compared to less active sluggish forms (Gray and Hall, 1930; Singh and Khanna, 1971; Benchoula et al., 2019). Grey and Hall (1930) reported that a sluggish fish *Lophius piscatorius* had only 5 to 6 mg blood glucose while an active salmon had upto 130 mg. Thus the findings of the present study clearly indicate that activity of the fishes, muscular exercise, food conversion ratios and energy mobility, increase blood glucose levels significantly.

Another reason for the higher serum glucose levels observed in carps cultured in domestic sewage may be due to large amounts of glycogen present in vital tissues of the fish particularly the liver. This was due to very active and increased processes of glycogenolysis, hyperglycemia and hyperlactemia which occurred in the experimental fishes. It is well known that the liver glycogen in fishes maintains the level of glucose in well fed animals and both are maintained and synthesized from non-carbohydrate sources also (Menten, 1927; Grey & Hall, 1930; Chavin and Young, 1970; Plummer, 1978; Chhabria et al., 2020). Thus the high carbohydrate metabolism associated with greater food conversion and enhanced glycolytic pathways clearly support the higher glycogen contents which was responsible for hyperglycemia and hyperlactemia in the fishes grown in domestic sewage waters. It is well known that starvation, under feeding and imbalanced diets cause depletion in glycogen levels in the fresh water cultured control fish tissues through affected carbohydrate metabolism. The blood glucose and tissue glycogen concentrations decrease under such conditions as observed by Kamra (1966); Joshi (1974); Ince & Thorpe (1975), Ghaly et al. (2005). Thus the data of fresh water cultured fishes of the present study are in full agreement with the above findings.

The carps grown in treated domestic secondary waste oxidation ponds do not face any kind of toxicological effect or of any kind of long term physiological stress which could damage the metabolic pathways causing depletion in sensitive biochemical components (Ali et al., 1988; Ali et al. 1991; Ali, 1992). The results of our findings are in corroboration with the very recent studies of Abdelhamid et al. (2015); Bhoi and Patole (2019); Grabicova et al., (2020). They have done experiments on carp fishes and channa cultured in sewage waters and found increased values of glucose and triglycerides.

CONCLUSION

It is concluded that the presence of high glycogen and glucose values in fishes cultured in secondary domestic sewage waters supports their good adjustment to that environment and reveals that physiochemical conditions of that ponds are not showing any negative impact on the biochemical parameters of the fishes grown there. Anyhow more research has to be done on fish culture experiments which are the biological treatment techniques for the purification of municipal or domestic effluent waters and also the health risks associated with the human consumption of fish grown in that water bodies.

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Antibiotic and Heavy Metal Resistance in Bacteria from Organs of Sewage Fed Farm Fishes

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ABSTRACT

Bacterial populations from organs (viz., liver, spleen, kidney and gill) of *Clarias batrachus* of the sewage fed water areas near IISCo slag disposal site, Dharampur on northern side of Damodar River, Asansol, West Bengal, India, were enumerated, followed by determination of resistance for antibiotics and heavy metals. Maximum resistance is shown against ampicillin (95%) and minimum against ciprofloxacin (5%). Most of the isolates exhibited an increasing order of tolerance for the metals (µg/mL) copper (200), cadmium (200), iron (400) and chromium (400), with minimum inhibitory concentration (MIC) ranging from <50 to 1600 µg/mL. A total of 100 bacteria have been successfully isolated from internal organs of *Clarias batrachus* (Aeromonas species (20%); *Escherichia coli*, (45%); *Bacillus* species (4%); *Pseudomonas aeruginosa* (6%), *Staphylococcus aureus* (18%) and coagulate-negative *Staphylococci aureus* (7%)). In terms of antibiotic susceptibility testing, each isolate was tested against 10 antibiotics. The multiple antibiotic resistance (MAR) index of the isolated bacterial ranged from 0.2-0.7. These observations indicate that the bacteria isolates are from a high risk source where antibiotics are frequently used, possibly from sewage effluents. Significant occurrence of bacterial population in organs of fish with high incidence of resistance for antibiotics and heavy metals may pose risk to fish fauna and public health.

KEY WORDS: *CLARIAS BATRACHUS*, ANTIBIOGRAM, HEAVY METAL RESISTANCE, MULTIPLE ANTIBIOTIC RESISTANCE.

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INTRODUCTION

Clarias batrachus, commonly called asian catfish is a threatened (Hossain et al., 2006; Ahmad et al., 2012; Roy et al., 2019), and critically endangered species (Binoy, 2010). It's a promising hardy fish, excellent nutritional profile and market price is high (Hossain et al., 2006; Goswami 2007; Debnath 2011).Tham et al., (2009) have reported heavy metal inhibitions by AchE from *C. batrachus*. Heavy metals are ubiquitous and persist as environmental pollutants that are introduced into the environment through anthropogenic activities, like

mining and smelting, also well as through irrigation and other sources of commercial waste. However, untreated or partially treated wastewaters introduce an enormous amount of contaminates particularly heavy metals into agricultural lands (Wang and Tao, 1998; Boateng et al., 2019). The existence of heavy metals within the environment represents a big and long-term environmental hazard since they're not biodegradable and have a tendency to accumulate in living organisms (Kobya et al., 2005; Liao et al., 2008; Genchi et al., 2020).

Indiscriminate use of various antibiotics has caused development of resistance for various antimicrobials and chemotherapeutic agents among the gut flora of homeotherms. Use of antibiotics will exert more selective pressure and resistant pathogens are going to be encontered more frequently (MacMillan, 2001; Priyadarshini et al., 2020). Resistance to antibiotics and metals occurs simultaneously when the genes specifying resistant phenotypes are located together an equivalent genetic element like a plasmid, transposon, or integron (Chapman 2003; Frost et al., 2005; Venner et al., 2009; McMillan et al. 2019). This present study was conducted to evaluate the antibiotic and heavy metal tolerance of bacteria obtained from internal organs of *Clarias batrachus*.

MATERIAL AND METHODS

Clarias batrachus were collected from the sewage fed water areas near IISCo slag disposal site, Dharampur on northern side of Damodar River at an altitude of 75 meters. Longitudinally it is at 86°55' East and latitudinally at 23°40' North Asansol, West Bengal, India. Total of 180 fish samples were collected from June to August 2019. 60 samples were collected at the first visit and was repeated three times. Fish were collected in sterile plastic bags with labelling from sites. Fish samples were transported to the laboratory in cooler box and tested on the same day. Fish samples were sacrificed to dissect out aseptically the liver, spleen, kidney and gills (Pathak and Gopal, 2005) placed in labelled bottle containing peptone water and buffered peptone water. The contents were homogenized and the homogenate from peptone water was streaked using a sterile loop on blood agar and MacConkey agar. Streaked plates were incubated at temperature (37 °C) aerobically for 24 hours. Homogenate in buffered peptone water was incubated for 24 hours at 37 °C. After incubation, 1 ml of the homogenate tranferred into Rappaport Vassiliadis (RV) agar and icubated for 24 hours at 37 °C. On third day, loopful of the sample containing bacteria was streaked on Xylose Lysine Deoxychocolate (XLD) agar and incubated for 24 hours at 37 °C (Markey et al. 2013).

The isolated bacteria were identified using morphological characteristics, Gram staining and biochemical tests (oxidase, motility, indole, citrate, lysine decarboxylase, urease. Triple sugar iron). The scheme of Cowan and Steel (1993) was followed for characterization and identification of strains, and the results were interpreted using Bergey's Manual of Systematic Bacteriology (Staley et al. 1989). Autoclaved fish and uninoculated media were used as negative controls. Different strains of bacteria obtained from MTCC Chandiagrh, India (Aeromonas 646, *Bacillus niacini* MTCC 8323, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, *Staphylocccus aureus* MTCC 2940) were used as positive control.

Antibiotic susceptibility testing was conducted consistent with Kirby and Bauer disk diffusion method (Bauer et al., 1966) by employing commonly used antibiotics (namely 30 µg of amikacin, 30 µg of amoxicillin, 10 µg of ampicillin, 30 µg of chloramphenicol, 5 µg of ciprofloxacin, 10 µg of gentamycin, 5 µg of levofloxacin, 10 µg of sparfloxacin, 25 µg of streptomycin and 30 µg of tetracyline). The colonies were transferred into agar plate. The swab was then streaked in three, different directions over the surface of plate of Mueller-Hinton Agar such that a uniform well spread out inoculum is achieved. After 18 hours of incubation at a specific temperature (37±1 °C) the plates were examined and the diameters of the inhibition zone was measured to the nearest millimeter. Inhibitions were measured and the result interpreted using Clinical Laboratory Standard Institute (2017).

The strain isolated was tested to four metals by Agar dilution method (Malik and Ahemad, 2006; Kinare and Shingadia, 2014). Stock solutions of 104 µg/mL were prepared by dissolving the precise quantities of the follwing metal salts: $CdCl_2$ (SRL), $K_2Cr_2O_7$ (SIGMA-ALDRICH), $CuSO_4$. 5H2O(SIGMA-ALDRICH) and $FeCl_3$ (MERCK) in water and sterilized. 20 mL of agar was poured into petri plates and therefore the volume of metal stock solutions was calculated by the formula: C1 × V1 = C2 × V2, where C1 is the metal concentration avaiable, V1 is the volume of stock solution used, C2 is that the concentration of metal in agar, and V2 is that the volume of agar. Then the isolated strains were streaked onto the medium-containing increasing concentrations of metal salts using sterile loops. Then, plates were sealed and incubated at 30 °C for five days. Plate-containing only agar was also inoculated and incubated to act as control. Rock bottom concentration of every metal at which no growth occurred in comparison to the control plates was considered as MIC value.

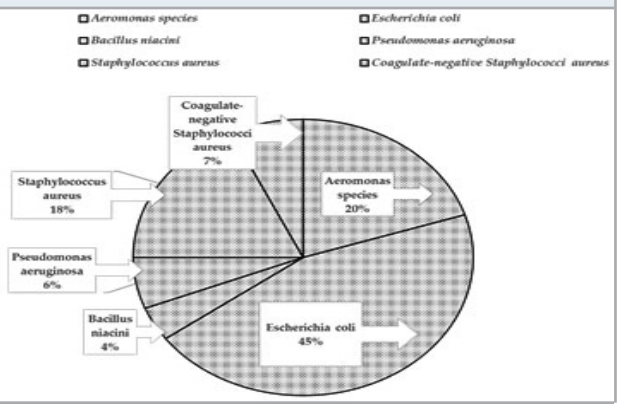
The multiple antibiotic resistance (MAR) index of isolates against tested antibiotics were determined as described by Krumperman (1983) using the formula: a/b, where "a" represents the number of antibiotics against which a particular isolate was resistant and "b" the total number of antibiotics used for test.

RESULTS AND DISCUSSION

The bacteria isolated were Aeromonas species, *Escherichia coli, Bacillus niacini, Pseudomonas aeruginosa*, *Staphylococcus aureus*, and coagulate-negative *Staphylococci aureus* which were identified using morphological properties, gram staining, and series of biochemical tests. Total 100 bacteria were isolated from

liver, spleen, kidney and gill. The bacterial load, i.e., total viable count, was found to be 5.62×10^4, 4.12×10^4, 2.30×10^4 and 1.76×10^4 c.f.u./mL in liver, spleen, kidney and gill of the experimental fish, respectively. The percentage prevalance of the isolated bacteria were *Aeromonas* species (20%); *Escherichia coli*, (45%); *Bacillus* species (4%); *Pseudomonas aeruginosa* (6%), *Staphylococcus aureus* (18%) and coagulase-negative *Staphylococci aureus* (7%). Prevalence rate of isolated bacteria are plotted in (Figure 1).

Figure 1: Prevalance rate of the isolated bacteria



The antibiotic resistance among random bacterial isolates from all four organs has shown a full range of resistance (0–100%) for ten common antibiotics of therapeutic and prophylactic uses among human beings, and in fish aquaculture. Resistance was found to be maximum among the isolates from spleen, kidney, and liver, while it was minimum among those from gill. Maximum average resistance was exhibited for ampicillin (95%) and tetracycline (75%) and minimum for ciprofloxacin (05%) (Table 1).

The heavy metal resistance percentage among bacterial isolates from fish organs presented in Table 2. The maximum tolerance, in general, was observed for chromium and iron (400 µg/mL), while it was minimum for copper and cadmium (200 µg/mL). The MIC value of isolates is presented in Table 3. Test isolates were also found to be tolerant to different concentrations of various toxic heavy metals as evidenced by their MICs ranging from <50 to 1600 µg/mL (Table 3). Except some gill isolated bacteria (ADCG14-ADCG21) the MAR index of all isolates show values higher than 0.2 (Table 4). All the isolate (except ADCG14-ADCG21) were multidrug resistant (resistant to three or more drugs). The trend of MAR index is alarming for the bacterial species isolated from the site.

Table 1. Antibiotic resistance (%) among bacterial isolates from fish organs

Antibiotics (µg/mL)	Fish organs				Average resistance
	Liver	Spleen	Kidney	Gill	
Amikacin (AMK) (30)	90	80	60	30	65
Amoxycillin (AMX) (25)	90	80	70	40	70
Ampicillin (AMP) (10)	100	100	90	90	95
Chloramphenicol (CHL) (30)	80	50	50	20	50
Ciprofloxacin (CIP) (5)	10	10	00	00	05
Gentamycin (GEN) (10)	50	40	20	10	30
Levofloxacin (LVX) (5)	50	40	10	00	25
Sparfloxacin (SPX) (10)	60	50	30	20	40
Streptomycin (STR) (25)	20	10	10	00	10
Tetracyline (TET) (30)	100	80	70	50	75

Table 2. Heavy metal resistance (%) among bacterial isolates from fish organs

Heavy metals (µg/mL)	Fish organs				Average resistance
	Liver	Spleen	Kidney	Gill	
Copper (200)	10	00	00	00	2.5
Chromium(400)	100	100	80	70	87.5
Cadmium (200)	30	20	10	00	15
Iron (400)	90	100	80	50	80

Table 3. MIC values for different heavy metals among bacterial isolates from fish organs

Heavy metals	MIC values (µg/mL) for different Fish organs			
	Liver	Spleen	Kidney	Gill
Copper	< 50	-	-	-
Chromium	1600	1600	800	400
Cadmium	100	50	< 50	-
Iron	1600	800	800	400

Contamination of river water with municipal sewage and industrial effluent results in the occurrence of pathogenic microorganisms, particularly fecal bacteria and toxic metals, above their maximum permissible limits (Chatterjee et al., 2010, Iloms et al., 2020). Fish in such water are exposed to these bacteria and metals, which bioconcentrate in different organs of fish. It has been observed to be maximum in liver and minimum in gills. Thus, it appears from these findings that soft tissues in massive organs are more prone to bioconcentration of bacteria, leading to incidence of infectious diseases among the aquatic fauna. This may be due to availability of more nutrients and lack of exposure to the surroundings.

Table 4. Code number assigned with organs from which the bacteria is isolated, antibiotic resistance Profile and multiple antibiotic resistance index of the isolated bacteria from site

Sl No	Code No assigned to isolate	Organs from Isolated	Antibiotic resistant Profile	Resistant to number of Antibiotics	MAR Index
1	ADCL1	Liver	AMK,AMX,AMP,TET,CHL,GEN	6	0.6
2	ADCL2	Liver	AMK,AMX,AMP,TET, CHL,LVX	6	0.6
3	ADCL3	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
4	ADCL4	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
5	ADCL5	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
6	ADCL6	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
7	ADCL7	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
8	ADCL8	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
9	ADCL9	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
10	ADCL10	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
11	ADCL11	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
12	ADCL12	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
13	ADCL13	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
14	ADCL14	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
15	ADCL15	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
16	ADCL16	Liver	AMK,AMX,AMP,TET, CHL,LVX	6	0.6
17	ADCL17	Liver	AMK,AMX,AMP,TET, CHL,LVX	6	0.6
18	ADCL18	Liver	AMK,AMX,AMP,TET, CHL,GEN	6	0.6
19	ADCL19	Liver	AMK,AMX,AMP,TET, CHL,GEN	6	0.6
20	ADCL20	Liver	AMK,AMX,AMP,TET, CHL,GEN	6	0.6
21	ADCL21	Liver	AMK,AMX,AMP,TET, CIP,GEN,STR	7	0.7
22	ADCL22	Liver	AMK,AMX,AMP,TET, CIP,STR	6	0.6
23	ADCL23	Liver	AMK,AMX,AMP,TET,CIP,LVX,STR	7	0.7
24	ADCL24	Liver	AMP,TET,LEV,GEN,STR,SPX	6	0.6
25	ADCL25	Liver	AMP,TET,LEV,GEN,STR,SPX	6	0.6
26	ADCS1	Spleen	AMP,AMK,AMX,TET,GEN,LVX	6	0.6
27	ADCS2	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
28	ADCS3	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
29	ADCS4	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
30	ADCS5	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
31	ADCS6	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
32	ADCS7	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
33	ADCS8	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
34	ADCS9	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
35	ADCS10	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6

Table 4 Continue

36	ADCS11	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
37	ADCS12	Spleen	AMP,AMK,AMX,TET	4	0.4
38	ADCS13	Spleen	AMP,AMK,AMX,TET	4	0.4
39	ADCS14	Spleen	AMP,AMK,AMX,TET	4	0.4
40	ADCS15	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
41	ADCS16	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
42	ADCS17	Spleen	AMP,AMK,AMX,TET, CIP,STR	6	0.6
43	ADCS18	Spleen	AMP,AMK,AMX,TET	4	0.4
44	ADCS19	Spleen	AMP,AMK,AMX,TET,CHL,SPX	6	0.6
45	ADCS20	Spleen	AMP,AMK,AMX,TET, CIP,STR	6	0.6
46	ADCS21	Spleen	AMP,CHL,SPX,CIP,STR	5	0.5
47	ADCS22	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
48	ADCS23	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
49	ADCS24	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
50	ADCS25	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
51	ADCK1	Kidney	AMK,AMX, AMP,TET	4	0.4
52	ADCK2	Kidney	AMK,AMX, AMP,TET	4	0.4
53	ADCK3	Kidney	AMP, CHL, SPX,TET	4	0.4
54	ADCK4	Kidney	AMK,AMX, AMP,TET	4	0.4
55	ADCK5	Kidney	AMP, CHL, LVX,TET	4	0.4
56	ADCK6	Kidney	AMK,AMX, AMP,TET	4	0.4
57	ADCK7	Kidney	AMK,AMX, LVX,TET	4	0.4
58	ADCK8	Kidney	AMK,AMX, LVX,TET	4	0.4
59	ADCK9	Kidney	AMX, AMP, GEN,TET	4	0.4
60	ADCK10	Kidney	AMK AMP, GEN,TET	4	0.4
61	ADCK11	Kidney	AMK,AMX, GEN,TET	4	0.4
62	ADCK12	Kidney	AMX, AMP, GEN,TET	4	0.4
63	ADCK13	Kidney	AMK,AMX, AMP,TET	4	0.4
64	ADCK14	Kidney	AMK AMP, CHL, SPX	4	0.4
65	ADCK15	Kidney	AMK, AMP, CHL,TET	4	0.4
66	ADCK16	Kidney	AMX, AMP, CHL,TET	4	0.4
67	ADCK17	Kidney	AMX, AMP, CHL,STR	4	0.4
68	ADCK18	Kidney	AMX, AMP, CHL,SPX,TET	5	0.5
69	ADCK19	Kidney	AMP, AMP, GEN,STR	4	0.4
70	ADCK20	Kidney	AMK, AMP, CHL, STR	4	0.4
71	ADCK21	Kidney	AMK, AMP, CHL, SPX,TET	5	0.5
72	ADCK22	Kidney	AMX, AMP, CHL, SPX	4	0.4
73	ADCK23	Kidney	AMK,AMP, CHL, SPX	4	0.4
74	ADCK24	Kidney	AMX, AMP, CHL, SPX,TET	5	0.5
75	ADCK25	Kidney	AMK, AMP, CHL, SPX	4	0.4
76	ADCG1	Gill	AMK, AMP,TET	3	0.3
77	ADCG2	Gill	AMX, AMP, TET	3	0.3
78	ADCG3	Gill	AMK, AMP, TET	3	0.3
79	ADCG4	Gill	AMX, AMP, TET	3	0.3
80	ADCG5	Gill	AMK, AMP, TET	3	0.3
81	ADCG6	Gill	AMX, AMP, TET	3	0.3
82	ADCG7	Gill	AMX, AMP, TET	3	0.3
83	ADCG8	Gill	AMK, AMP, TET	3	0.3
84	ADCG9	Gill	AMX, AMP, TET	3	0.3
85	ADCG10	Gill	AMX, AMP, TET	3	0.3
86	ADCG11	Gill	AMK, AMP, TET	3	0.3
87	ADCG12	Gill	AMX, AMP, TET	3	0.3
88	ADCG13	Gill	AMK, AMP, TET	3	0.3
89	ADCG14	Gill	AMK, AMP	2	0.2
90	ADCG15	Gill	AMX, AMP	2	0.2
91	ADCG16	Gill	AMX, GEN	2	0.2

Table 4 Continue

92	ADCG17	Gill	AMX, GEN	2	0.2
93	ADCG18	Gill	AMP, GEN	2	0.2
94	ADCG19	Gill	AMP, SPX	2	0.2
95	ADCG20	Gill	AMP, CHL	2	0.2
96	ADCG21	Gill	AMP, CHL	2	0.2
97	ADCG22	Gill	AMP, CHL, SPX	3	0.3
98	ADCG23	Gill	AMP, CHL, SPX	3	0.3
99	ADCG24	Gill	AMP, CHL, SPX	3	0.3
100	ADCG25	Gill	AMK, AMP, SPX	3	0.3

Bioconcentration of aquatic bacteria such as coliforms, streptococci, and aeromonads in gut, liver, and muscles of tilapia fish grown in a sewage-contaminated pond has also been noticed (Fattal et al., 1993; Wamala et al., 2018). The resistance exhibited for ciprofloxacin, levofloxacin, streptomycin and gentamycin is a signal of the effectiveness of broad-spectrum antibiotics of the present generation. With these observations it appears that the source of the problem of antibiotic resistance in riverine ecosystems is fecally contaminated water, and fish populations in them plays important role in creating resistance. Antibiotic resistance patterns in the bacterial population in an aquatic ecosystem have been found to be useful in identifying non point sources of fecal pollution (Wiggins et al., 1999; Labrador et al., 2020).

The occurrence of resistance for common antibiotics is, further, an indication of indiscriminate use of these antibiotics, leading to constraint in antimicrobial therapy for infectious diseases. The loss of antibiotic susceptibility among the aquatic bacteria has been observed to be affected to a considerable extent by the physicochemical qualities of water and seasonal variations (Pathak et al., 1993). In addition to assessment of loss of antibiotic susceptibility, the test isolates were also found to be tolerant to different concentrations of various toxic heavy metals as evidenced by their MICs. The isolates from visceral organs, i.e., spleen, kidney, and liver, exhibited maximum resistance for ampicillin, tetracycline, and amoxicillin with the highest tolerance for iron and chromium; while the isolates from gills showed minimum resistance for ampicillin and tetracycline with rather low tolerance for cadmium and copper.

These observations indicate that visceral organs provide better conditions for bacterial growth and biological activity than exposed organs such as gill. Increase in the MIC of toxic metals as well as antibiotic resistance among aquatic bacterial populations is also an indication of risk to the safety of the aquatic ecosystem, fish fauna, and ultimately human health. MAR values were ranging from 0.2 to 0.7. MAR indexes of the present work revealed that bacteria from locally raised fish may has been exposed to test antibiotics. McPhearson et al., 1991 reported that the MAR index of bacteria from a catfish pond, near a river where antibiotic was commonly used as treatment, was as high as 0.76.

Currently, local fish farmers employ amoxicillin to treat fish diseases. The present results proven that amoxicillin is no longer effective, since maximum bacterial isolates were sensitive to it. It appears that the emergence of resistance is also influenced by the physicochemical characteristics of water and several environmental factors including hospital and *aquaculture waste disposal* (Rhodes et al., 2000; Iwu et al., 2020) along with the form and bioavailability of metals in the ecosystem. Resistance also develops from a non-specific mechanism with gene regulation of plasmids and chromosomes which may be heritable due to the presence of a resistance factor (R-factor) among the aquatic bacterial population (Silver and Walderhaug, 1992). The infections caused by the pathogenic bacteria with R-plasmids may pose a risk of therapeutic problems to public health and fish population. Thus, the water bodies with antibiotic and metal resistant bacteria serve as an environmental reservoir and source for the development of this trait among opportunistic pathogens and constitute a significant public concern.

Therefore, such studies should be considered for the selection of antibiotics in dealing with water-borne diseases, particularly among fishermen and fish consumers. These findings indicate that sewage and industrial pollution are responsible for the emergence of bacterial resistance and deterioration of water quality, along with risk to biodiversity of the hydrobionts and the human health.

Conflict of Interest: We declare that we have no conflict of interest

Ethical Approval: This article is not under consideration or published elsewhere. Ethical clearance for the study was obtained from IAEC, Approval No. 17/IAEC (05)/ RNLKWC/2019, Dated 27.07.2019.

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Functional Activity of the Cardiorespiratory System and the General Level of Physical Capabilities Against the Background of Regular Physical Exertion

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ABSTRACT

For men of the second adulthood who had myocardial infarction, a very characteristic decrease in their body's resistance to hypoxia, is a weakening ability to tolerate physical activity and a decrease in the functional capabilities of the cardiovascular system. The tested author's scheme of physiotherapy exercises, including morning exercises, therapeutic exercises during the day, breathing exercises, walking upstairs to 2 floors and dosed walking along a horizontal plane 300 meters, provided for men aged 45-60 years who had myocardial infarction and had increase in their physical capabilities. The study involved 43 men aged 45 to 60 years who had a small focal myocardial infarction in the posterior or anterolateral walls of the left ventricle 5-6 days ago without signs of heart failure. They were randomly divided into two comparable groups - the first experimental group (21 people) and the second experimental group (22 people). The control group consisted of 34 clinically healthy men aged 45-60. Against the background of its use, it was found that they managed to significantly increase the body's resistance to physical exertion and hypoxia, as well as significantly increase the functional activity of their cardiovascular system. The effectiveness of the author's scheme of therapeutic physical culture was significantly higher than the results of applying the traditional scheme in terms of rehabilitation of this patient population. It was also ensured that the volunteers had all the indicators taken into account to the level of clinically healthy people.

KEY WORDS: PHYSICAL THERAPY, MYOCARDIAL INFARCTION, HEART, PHYSICAL ACTIVITY, REHABILITATION, PHYSICAL CAPABILITIES.

INTRODUCTION

Currently, cardiovascular diseases remain highly prevalent among the population of developed countries and are the leading cause of death worldwide, (Medvedev, Kumova, 2007; Skoryatina, Medvedev, 2019). The main share in the prevalence of disease is ischemic heart disease and in particular myocardial infarction. Its frequency in recent years in the world and in Russia is steadily growing. Most commonly found in men – on average 3 to 5 cases per 1000 population (Kovyazina, 2016). Myocardial infarction is still considered a life threatening disease, can lead to serious disability due to a significant deterioration of health and the formation of social exclusion (Strezhneva, 2017; Medvedev, 2018a).

For this reason, much attention is paid to aspects of rehabilitation after myocardial. The basis for the restoration of health and return patients to home, public and professional activities after myocardial infarction is a physical rehabilitation (Kozyreva et al., 2018). From the timeliness of its beginning, the sequence of its implementation and successful use in the process of modern science-based programs on the

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overall effectiveness of rehabilitation in these patients (Medvedev, 2018b; Boldov et al., 2018).

To ensure the effectiveness of physical rehabilitation after myocardial infarction possible only through integrated use of various means of medical physical culture (Stepanova et al., 2018). Currently, many aspects of its use after myocardial infarction remain controversial. This dictates the need to clarify the schemes of application of medical physical culture in this category of patients during their physical rehabilitation. The goal of the present work is to evaluate the effectiveness of the author's prescribed technique of medical physical culture in the course of rehabilitation of patients after myocardial infarctions.

MATERIAL AND METDODS

The present study was approved by the local ethics committee of the Russian State Social University on September 15, 2017 (Protocol No 11). The study involved 43 men aged 45 to 60 years who had a small focal myocardial infarction in the posterior or anterolateral walls of the left ventricle 5-6 days ago without signs of heart failure. They were randomly divided into two comparable groups - the first experimental group (21 people) and the second experimental group (22 people). The control group consisted of 34 clinically healthy men aged 45-60.The first experimental group was engaged in physical therapy according to the traditional scheme, including therapeutic exercises lasting about 30 minutes a day and walking along the corridor and stairs on one floor for 20 minutes a day.

The second experimental group was engaged in the therapeutic physical culture scheme developed by the author, including morning exercises, therapeutic exercises during the day and breathing exercises for a total duration of 1 hour, walking with stops upstairs to 2 floors and dosed walking with stops along the horizontal plane for a gradually increasing distance from 300 meters to 2000 meters. At the beginning, overcoming the daily distance was allowed in 2-3 doses. The total duration of walking lessons per day in the second group was at least 70 minutes. The duration of classes according to the traditional and author's schemes of therapeutic physical culture in both groups was 1.5 months.

Table 1. The results of the rehabilitation of men after myocardial infarction Legend: p - significance of differences in outcome and control, p1 - significance of dynamics of indicators during rehabilitation, p2 - significance of differences in rehabilitation results in both groups of patients.

Indicators	Experimental group 1, M±m, n=21		Experimental group 2, M±m, n=22		Control group, M±m, n=34
	start of observation	end of observation	start of observation	end of observation	
seconds	22.7±0.62 P<0.01	34.2±0.75 P_1<0.01	23.4±0.42 P<0.01	45.8±0.37 P_1<0.01 P_2<0.01	49.9±0.72
Genchi test, seconds	17.1±0.42 P<0.01	24.2±0.29 P_1<0.05	18.1±0.35 p<0.01	32.9±0.48 P_1<0.01 P_2<0.01	39.0±0.51
Orthostatic test, beats per minute	18.5±0.36 P<0.01	16.8±0.41 P_1<0.05	19.1±0.27 P<0.01	12.4±0.34 P_1<0.01 P_2<0.01	12.0±0.26
Test 6 minutes walk, meters	375.0±3.14 P<0.01	827.4±2.28 P_1<0.01	374.0±4.07 P<0.01	1061.3±6.18 P_1<0.01 P_2<0.05	1145.4±7.62
Systolic blood pressure, mmHg.	148.3±0.92 P<0.05	139.1±0.87	146.6±0.72 P<0.05	130.4±0.89 P_1<0.05	128.9±0.68
Diastolic blood pressure, mmHg.	86.8±0.61 P<0.05	80.0±0.57	85.4±0.70 P<0.05	73.7±0.62 P_1<0.05 P_2<0.05	74.0±0.53

RESULTS AND DISCUSSION

The initial state of the indicators in both observation groups turned out to be comparatively impaired and had no statistically significant differences between themselves. When taking into the study, the indicators of the applied samples in patients were comparatively inferior to the level of control - the breath holding time in the Stange sample was more than 2 times, in the Genchi sample more than 2 times. This was accompanied

by a similar increase in heart rate in an orthostatic test outcome in both groups of patients. The distance that patients were able to overcome in the test of 6 minutes of walking at the end turned out to be almost 2.8 times less than this distance in the control group in both experimental groups. Moreover, the levels of systolic and diastolic blood pressure in the outcome in both groups of patients exceeded the control level by approximately 15.6% and 13.5%, respectively.

As a result of the healing effects in both groups, men who had myocardial infarction had a positive dynamics of all the studied parameters. Their changes in the second experimental group were more preferable, which made it possible to approach all considered indicators to the level of control. At the same time, occupations with therapeutic physical culture according to the traditional method contributed to a more modest improvement in the functional state of the cardiorespiratory system and a less pronounced increase in the physical capabilities of patients. The results of the studies are shown in the table 1.

Regular muscular load according to the traditional scheme provided for an increase of the sample Rod at 50.7%, which brought the figure to a lower level of control of 45.9%. Lessons of medical physical culture on the authors' scheme helped to increase the delay time breathing in this sample, 95.7%, ensuring the normalization of this index in the second experimental group. The application of therapeutic physical culture on the traditional pattern increased the provisional rate in the sample of genchi 41.5%, but the achieved results inferior to the control level at 61.1 per cent. Sessions with patients in a medical physical culture according to the author's scheme provided the normalization of the value of this sample due to an increase of 81.8%.

Physical activity on the traditional pattern was followed in the first experimental group the reduction in the rate orthostatic test by 10.1%, but the achieved rate 40.0% inferior to the level of control. Classes conducted with patients in the second experimental group was provided access indicator of the orthostatic test on the control level as a result of his demotion to 54,0%. Classes of therapeutic physical culture has improved the overall physical fitness, which was judged by the test results of 6-minute walk on the traditional scheme 2.2 times (the result is inferior to the control 38.4%), on the author's schema in 2.8 times, which ensured in this case, his normalization.

Therapeutic physical culture, conducted by the traditional scheme, to provide some decrease in blood pressure, whereas the output level of the control group was only in result of application of the author's scheme of medical physical culture.At present, the prevalence of myocardial infarction, ischemic necrosis of a part of the heart muscle that develops as a result of coronary artery thrombosis, remains very high (Medvedev, 2018c). Its occurrence is associated with the death of some cardiomyocytes in the blood supply zone of the occluded artery with the subsequent development of a scar at this site (Vatnikov, Rudenko et al., 2019). Due to the occurrence of cardiomyocyte deficiency in this place, often the contractility of the heart decreases in post-infarction patients, which is the reason for the decrease in their working capacity and the appearance in some of them of varying severity of heart failure (Medvedev, 2018d).

In this regard, it is very important to continue the search for approaches to improving the physical rehabilitation of patients after myocardial infarction, aimed at increasing the degree of restoration of their performance (Vorobyeva, Medvedev, 2019). Moreover, an especially important role in this process is traditionally assigned to increasing the reserve capacity of the heart in such patients. It is recognized that this problem can be most effectively solved by the early activation of these patients and the active use of dosed physical exercises in them (Medvedev, 2019). Early muscle activation contributes to the development in their heart, especially around the necrosis zone, of full-fledged adaptation processes, the strengthening of the forming scar and promotes a persistent positive psychological attitude in patients to recover (Lenchenko, Vatnikov et al., 2019).

More active muscle activity within the framework of the author's scheme of therapeutic physical culture provided greater stimulation of the functional activity of systems and organs, providing higher opportunities for its implementation than traditional classes of therapeutic physical culture. There is no doubt that in the process of its implementation in the second experimental group, more significant changes developed on the part of the nervous, cardiovascular and respiratory systems, metabolic processes and blood composition (Bikbulatova, 2018c). Moreover, the positive dynamics of metabolic processes in the central nervous system, which optimize the coordination of changes throughout the body, played an important role in the upcoming functionally beneficial changes. This contributes to the rationalization of movements and the formation of a dynamic stereotype that helps general recovery (Medvedev, 2018i).

More active muscular work according to the author's scheme, apparently, increased the level of excitability in the nerve cells of the coordinating centers to a greater extent than physical therapy by traditional methods. At the same time, against the background of the author's technique, inhibition processes were more pronounced in the central nervous system, due to which only in this case a physiologically favorable equilibrium was established between excitation and inhibition (Bikbulatova et al., 2018).Apparently under the influence of nervous impulses originating from the Central nervous system, in the course of medical physical culture by the author's scheme in the muscles maximally increased biochemical and biophysical processes that provide reduction. The more successful of muscle activity inevitably there was a significant positive functional shifts in the work of all internal organs (Medvedev, 2018j).

The obtained results give reason to believe that the classes of medical physical culture by the author's scheme has been able to provide patients with myocardial infarction develop greater resistance to hypoxia. Apparently, this is based on a significant increase in the number of mitochondria in their cells and the increase of enzyme activity, primarily aerobic glycolysis. These changes naturally resulted in more pronounced increase total physical capacity, which was judged by the test results of 6-minute walk. Achievement in the application of the copyright scheme a higher level of physical capacity in post-infarction patients provided they have a physiologically beneficial adaptation to daily activity (Medvedev, 2018g).

Based on literature data it is possible to think that the basis of the achieved effect was the development of physiological hypertrophy part of skeletal muscles and thickening of cardiomyocytes, primarily around post-infarction scar, due to the accumulation of protein and mitochondria. It is stimulated in heart patients metabolism, optimizing it processes of depolarization and repolarization. At the same time engaged in a medical physical culture according to the author's scheme, apparently, has resulted in a substantial weakening of the tone of smooth muscle in the walls of arteries of medium caliber. These changes have led to the application of the copyright scheme of medical physical culture resistant to physiologically beneficial to the overall hemodynamic decrease in systolic and diastolic blood pressure (Bikbulatova, 2018b).

It may be thought that the achievement of a more pronounced general recovery of patients who had myocardial infarction during the application of their own methods of physical therapy was possible as a result of strong and gentle stimulation of metabolic processes in the skeletal muscles and myocardium, causing accelerated scarring in the necrosis zone and activating compensation mechanisms in the myocardium around it, surpassing those against the background of dosed muscle loads in the aerobic regimen involving several muscle groups traditional scheme.

CONCLUSION

The functional features of men aged 45-60 who have had myocardial infarction are a decrease in the resistance of their cardiovascular system to hypoxia and a decrease in their body's ability to tolerate physical activity. The developed author's scheme of therapeutic physical culture was able to increase in men aged 45-60 who had myocardial infarction, resistance to physical activity and to hypoxia of their cardiovascular system and the whole organism. The effectiveness of the use of the author's scheme of therapeutic physical culture significantly exceeded the effectiveness of the traditional scheme, since only in the first case, all the considered parameters in post-infarction patients were brought closer to the level of clinically healthy people.

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A Survey on Time Management Among Students of Higher Education

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ABSTRACT

Time management comprises one of the most customary points in the field of learning and study techniques, involving a focal situation in the courses and various handbooks on study aptitudes. Time management can be characterized as the way toward arranging and performing pre-decided exercises over a particular length of time, fundamentally with the objective of expanding profitability, adequacy and effectiveness. It requires a cautious adjusting between professional life, social activity, hobbies or other activities. There are two dominating perspectives in presence regarding time management - multi-active time view and linear-active time view. The latter spotlights on a more prominent number of errands on the double, though, linear-active time view centers around each specific assignment in turn. Time management successfully goes about as a strategy for seat stamping progress and positively affects scholarly execution. This study aims at studying the effectiveness in time management among college students of various courses. In this experimental research, 160 college students were selected across various colleges in Chennai. Questionnaires were prepared and distributed to the participants through an online portal. The descriptive statistics was carried out and chi square test was used and p value was calculated through SPSS software. The results have shown that there is significant statistical difference (p value < 0.05) with respect to time management. The results show that 36.8% of the female students spend more time in studying than the male students, 50.6% of the female students concentrated for more than half the class time during the lecture class than the male students and 49.3% of the female students felt that preparing timetable before exams for studying will improve their grades.

KEY WORDS: TIME MANAGEMENT, STUDENTS, EXAMINATION, PERFORMANCE, RESULTS.

INTRODUCTION

The progress from higher secondary schooling to college speaks a significant change in the lives of youngsters. An exceptionally organized profoundly directed home condition to moderately unaided, unstructured ground conditions is a drastic change for youngsters. Despite the fact that numerous undergraduates have similar curricular and extracurricular demands on their time as they did before going to college, they are not required to truly be in a solitary structure for 8 hours every day nor are their recreation exercises observed by guardians. These external constraints do not have a role to play in their daily life activities and are free to choose their work as time permits which include devoting time for studies, socializing, engaging themselves in their passionate works, or other entertainment activities.

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The age clearing its path towards the beginning of the second decade of the 21st century has an exceptional number of alternatives regarding how to manage their recreation or leisure time.

Their adolescence matched with the advocacy of new media served to duplicate the number of encounters. Academic competence is related to the student's capacity to deal with their investigation load and is utilized to survey if the students can deal with the exam material in the curriculum. It additionally gives a sign of whether the educational program is intriguing enough for students to make the most of their classes. Academic fitness has appeared to influence the student's scholarly exhibition and an undergraduate with better scholastic skills would likely have better scholarly performance (Kleijn, Ploeg and Topman, 1994).In this examination, scholastic competency is characterized as the capability of students as for the substance instructed during courses over the past scholarly year and their capacity to comprehend the course material. Another factor is related to a student's scholastic execution is test competence which reflects how higher education students adapt to the measure of study material for examinations (Indries and Bochis, 2019).

It alludes to challenges related to dealing with the measure of study material for an assessment and in getting ready for them. Test competency is operationally characterized as any undergraduate or postgraduate's capacity to oversee and adapt to the measure of study material for assessments as well as tests. Vital considering strategies may assist higher education students with accomplishing a high GPA. Vital considering is characterized as the information and utilization of compelling investigation abilities or procedures by students. There are numerous proficient examination methods that could be utilized by higher education students dependent on the learning environment. These investigation techniques incorporate Know-Want-Learn (K-W-L), Survey-Question-Read-Recite-Review (SQ3R), summarizing and note-taking, utilizing graphics, and self-questioning (Wiles, 1968; Brown and Day, 1983; Ogle, 1986; Armbruster, Anderson and Meyer, 1991; Burke, 2002; Pearson et al.., 2016).

Extensive course stacks and the comprehensive data shrouded in the present pharmacy educational programs require the utilization of viable examination methodologies for scholarly success. Time management aptitudes are additionally imperative to scholarly achievement. Time management has been characterized as groups of social ranges of abilities that are significant in the association of study/course load (Mchargue, 1977; Lay and Silverman, 1996; Deshler et al., 2001).Time management skills incorporate exercises performed by students, for example, arranging ahead of time, organizing work, test arrangement, and following schedules (Kirschenbaum and Perri, 1982). Higher scholarly execution might be accomplished by adjusting the time management domain and study strategies effectively (Powell, 2004; Entwistle and Ramsden, 2015).

In this investigation, the time management area was operationalized as the capacity of higher education students to shuffle relaxation and study time to plan for their examinations (Hembree, 1988; Sarason and Sarason, 1990; Seipp, 1991). Test anxiety is adversely connected with scholarly performance Test tension is a set of responses like worry, depression, nervousness, task-irrelevant cognitions(Sarason, 1980; Zeidner, 2006), etc, to a class of stimuli arising from an individual's experience of assessment or testing Test nervousness in this study was defined as the reaction to stimuli that are associated with an individual's experience of testing or evaluative situations. Various programs that include the methods to reduce or improve the stress levels will help students to improve scholastic execution by decreasing feelings of anxiety. Demographic details, for example, student's age, sexual orientation, ethnicity, and marital and work status may likewise impact a student's uneasiness levels (Lapp, 2011).

The essential target of this investigation was to investigate the impact of scholarly ability, test capability, time management, vital examining aptitudes, and test tension on students' scholastic execution. Early detection and understanding reasons for scholastic disappointment may enable certain students to perform better if satisfactory direction on progress is given proficiently (Powell, 2004; Entwistle and Ramsden, 2015). Academic stress is supposed to be an upsetting pattern among the undergraduate students. Academic stressors affecting the college students include the lifestyle with respect to their health; time invested for studying, their financial limitations, self-imposed factors and the major criteria which is the time. These stressors were categorized based on the student's perception of an extensive knowledge base and the perception of inadequate time required for it (Macan et al.. 1990).

Poor time management behaviours, for example, not apportioning time appropriately or last-minute packing for tests, have been as often as possible examined as a wellspring of stress and helpless scholastic execution. As sensible as these desires seem to be, just a couple of experimental contemplates have endeavoured to test these connections. With an end goal to address this lack, we planned our examination to evaluate connections of higher education students' time to the board to self-revealed scholastic execution and different feelings of pressure. The essential proposals are to recognize needs and rank them with respect to their significance or need, and afterward apportion time and assets appropriately. Different tips include:.

Try to deal with each bit of paper just a single time, delegate work, and consistently ask yourself "What is the best utilization of my time at this moment?" Of the constrained exploration that has managed time on the board, most investigations have concentrated on the impacts of various kinds of guidance on saw pressure and conduct. Despite the fact that this past examination experiences methodological blemishes, the discoveries appear to demonstrate that preparation can change

how one invests energy (Powell, 2004; Entwistle and Ramsden, 2015).

Undergraduate students report encountering scholarly worry at unsurprising occasions every year with the best wellspring of scholastic pressure coming about because of taking and reading for tests, grade rivalry, and the enormous measure of substance to ace in a modest quantity of time. At the point when stress is seen contrarily or experienced unnecessarily, students also face physical and mental weakness (Misra and Castillo, 2004). At the point when stress is seen contrarily, or when it becomes exorbitant higher education students experience physical and mental impedance. Strategies to diminish worry by higher education students regularly incorporate successful time with the executives, positive reappraisal, social help, and commitment to positive relaxation exercises.

Relaxation fulfilment is characterized as the constructive sentiment of satisfaction one sees because of addressing individual needs through recreation exercises. The administration aptitudes that make an interpretation of inspiration into the real world and adapting to mental imperatives between exercises are additionally significant (Jackson, Crawford and Godbey, 1993). These official capacities incorporate time the executives and objective directedness, (Goodale and Witt. 1980). Seen absence of time is frequently identified with hesitance to take part in life exercises. Time management is a self-regulatory expertise that includes observing the most effective approaches to utilize time.

MATERIAL AND METHODS

Sample collection: A total of 20 questions with multiple choice options (Annexure 1) were distributed among 160 college students studying various courses from different universities across Chennai. The questionnaire included questions regarding study time, recreation and leisure time and also various other constraints such as concentration time during lecture classes, preparing timetables to study for exams.

Sampling Method: In the present study, the sampling method used is a random sampling method. Data Collection And Tabulation- The responses of the study population were retrieved in the google sheets and then tabulation of the data finally and the question comparison was done. The representation of the data is through the bar graph.

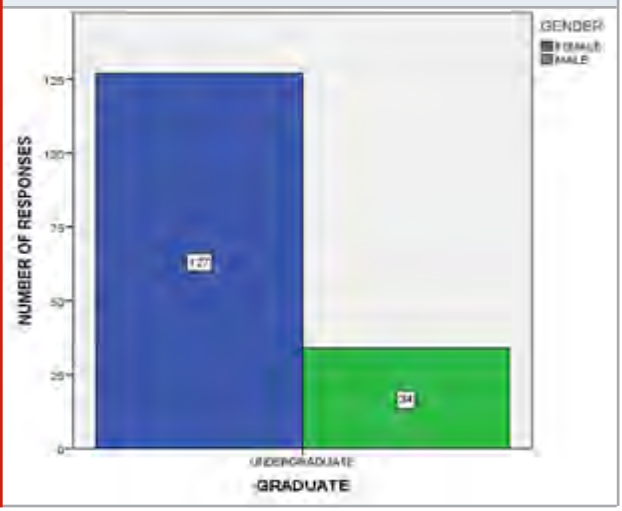
Annexure 1

QUESTIONNAIRE

1. Name and Year of study?
2. Undergraduate or postgraduate?
3. Gender: Female/ Male
4. Course that you are studying?
5. How often do you study on a regular basis? A: 1 hour/ less than 1 hour/ more than 1 hour
6. How often do you devote your time for leisure activities? A: 1 hour/ less than 1 hour/ more than 1 hour/ intermittently
7. After 8 8hours of college time, how often do you feel exhausted? A: On a regular basis/ after the practical sessions/ after hectic lectures/ none of the above
8. How well can you concentrate in a lecture? A: Less than ¼ the class time/ ½ of the class time/ for the whole lecture class.
9. Are you able to cope up with the stress induced during exams? A: Yes/ no
10. How often do you think that exams create mental stress? A: Sometimes/ often/very often
11. During exams, how many hours do you spend in studying? A: 8hrs/ less than 8 hrs/ more than 8hrs
12. How often do you think that you could have done better if you would have invested more time in studies? A: Sometimes/ often / very often
13. Do you agree with the statement that exams create, "Divide and Rule"? A: Agree/ strongly agree/ disagree/ strongly disagree
14. How often are you afraid of getting grades lower than your friend? A:Sometimes/ often/ very often
15. How often are you likely to go out on a daily basis for refreshments and recreation? A: More than 3 hrs/ less than hrs/ intermittently take breaks
16. How often do you feel that studies could have been more interesting when coming to conventional classroom learning? A: Sometimes/ often/ very often
17. Do you agree that conventional classroom learning provides a better learning experience than the recent online learning? A: Agree/ strongly agree/ disagree/ strongly disagree
18. How often do you invest your time for socialising with your friends, family and others? A: Sometimes/ often/very often/ intermittently
19. Do you agree that professional courses need more time investment towards studies than other courses? A: Agree/ strongly agree/ disagree/ strongly disagree
20. Do you agree that preparing a timetable will help you devote more time for studies? A: Agree/ strongly agree/ disagree/ strongly disagree
21. Why do you think that time management is essential for college students? Comment briefly.

Statistical Analysis: The statistical software used IBM SPSS V23.The statistical test used is Chi square test (p value).The software helps to describe the data and provide descriptive statistics, frequencies and percentage analysis for categorical variables and SD was used for continuous variables to find significant difference between the bivariate samples of independent groups. Inclusion Criteria: Selection criteria include age greater than or equal to 18 years and undergraduate and postgraduate students of medical, dental profession and students of other courses who are willing to participate were included in this study. Exclusion Criteria: Students who are willing to participate and other students were excluded from this study.

Figure 1: Bar graph shows the gender distribution of the study population. It shows that 79.3% of them were female (Blue) and 21.25% of them were males (green).



RESULTS AND DISCUSSION

The results showed that all (100%) participants were undergraduate students and data collected were tabulated in google sheets and analysed in SPSS version 23. The figure 1 showed that the majority of the study population were females (79.3%) than males (21.25%). In our study population, the majority were undergraduate BDS students (73.75%), followed by 16.25% were students from other professional courses and 10.6% of MBBS students (figure 2). It was found that only 43.75% of the students study daily for more than 1 hour. Majority of the students study only for 1 hour or less 1 hour daily (figure 3). As in figure 4 around 50% of the students felt exhausted after normal class hours, remaining felt exhausted after hectic lectures and practical sessions.

Similarly, 48.75% of the students were taking more than 1 hour for leisure activities and the remaining were taking leisure activities for exactly 1 hour or less than that (figure 5). In figure 6 it was shown that only 17.5% can concentrate during the whole lecture class irrespective of the class hours, 19.375% of the study population could concentrate only for less than half of the lecture time and a majority of the study population opted that they could concentrate for more than half of the lecture time but not for full class time. This was well correlated with a study in which the authors discussed the focal points related to successful time management in which the instructions were allegedly various. In addition, inefficient time management practices, for example, inappropriate designation of time for work tasks, packing for tests and neglecting to comply with time constraints set by scholastic staff are often referred to as a significant source of stress and inefficient scholarly execution (Middendorf and Macan, 2002).

Figure 2: Bar graph shows the profession of the study population where 73.75% of them were BDS students, 10.6% were MBBS students and 16.25% belonged to other professional courses.

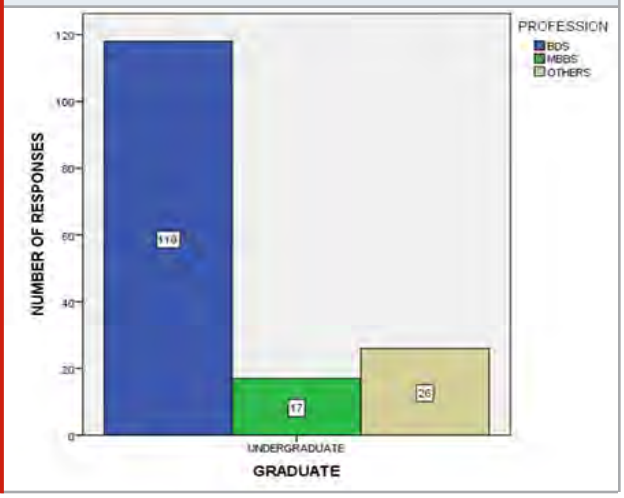


Figure 3: Bar graph shows the frequency distribution of student's study time on a daily basis. It shows 43.75% of the students studied for more than 1 hour, 16.8% of the students studied for less than 1 hour and 40% of the students studied for exactly 1 hour.

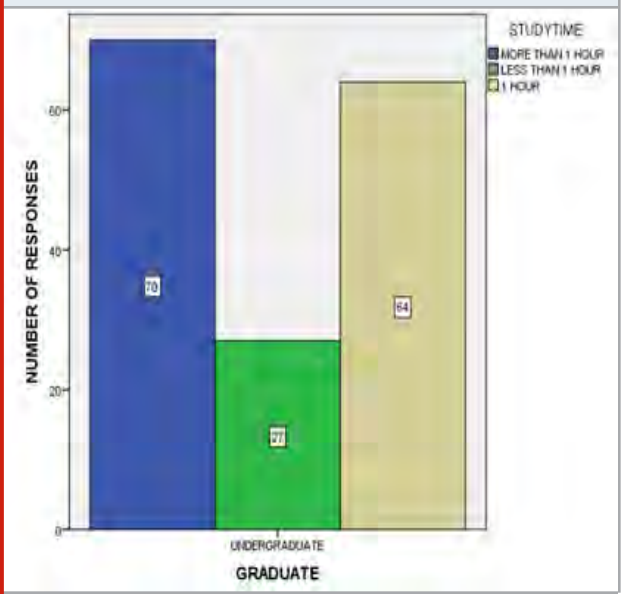


Figure 4: Bar graph depicts the frequency distribution of students getting exhausted on a daily basis. It shows that 23.75% of the students felt exhausted after hectic lectures, 49.375% of them felt exhausted even after the normal class days, 15% of the students felt exhausted after the practical sessions due to the workload experienced by them and 12.5% of the students did not get exhausted to the above said reasons.

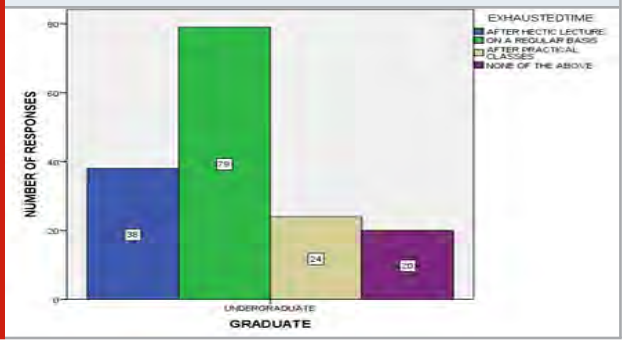


Figure 5: Bar graph shows the frequency distribution of students taking leisure time on a routine basis. It shows that 48.75% of the students take more than 1 hour for leisure activities, 39.375% of the students opted for less than 1 hour of leisure time and 12.5% of the students took exactly 1 hour of leisure time on a daily basis.

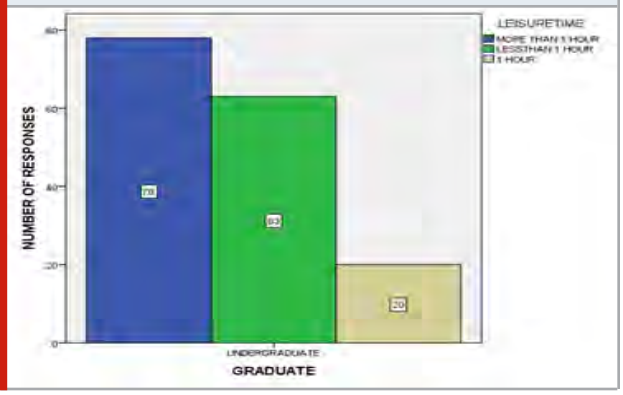


Figure 6: Bar graph shows the frequency distribution of students concentrating in a lecture conducted in their respective colleges. It shows that 17.5% of the students could concentrate for the whole of the lecture class, 63.75% of the students could concentrate for more than half the lecture time but not for full class and 19.375% of the students could concentrate only for less than half the lecture time.

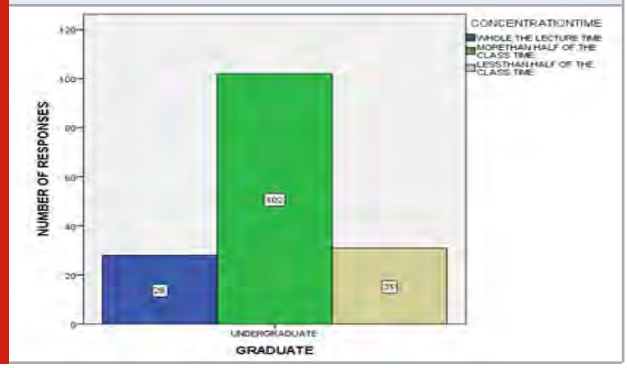
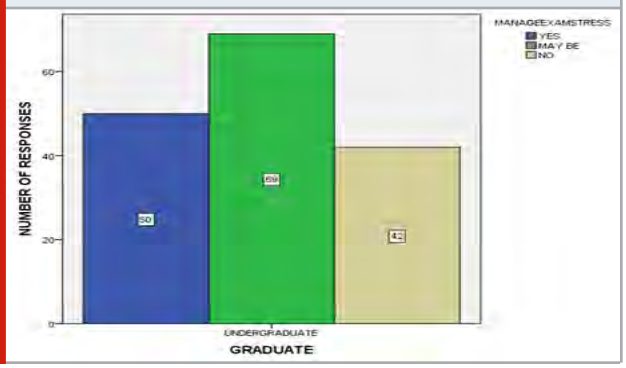


Figure 7: Bar graph shows the frequency distribution of students agreeing to manage their exam stress. It shows that 31.25% of the students agreed that they managed exam stress, 43.125% of the students that they could sometimes manage but fail to do so the rest of the time and 26.25% of the students agreed that they were not able to manage the exam stress.



In relation to the exam and the study holidays (figure 7 and 8), 31.25% of the students agreed that they managed exam stress, 43.125% of the students that they could sometimes manage but fail to do so the rest of the time and 26.25% of the students agreed that they were not able to manage the exam stress. 26.8% of the students have opted that they often have stress during exam time, 23.12% of the students have agreed that they always have exam stress, 43.75% of the students have said that they experience exam stress only sometimes, 5% of the students rarely experienced stress during exams and 1.87% of the students never experienced exam stress. During study holidays, 48.12% of the students' study for more than 8 hours during the exam time, 34.7% of the students' study for less than 8 hours during exam time and 18.2% of the students studied exactly 8 hours a day during exam time (figure 9).

Figure 8 Bar graph shows the frequency distribution of how often the students have stress during their exam. 26.8% of the students have opted that they often have stress during exam time, 23.12% of the students have agreed that they always have exam stress, 43.75% of the students have said that they experience exam stress only sometimes, 5% of the students rarely experienced stress during exams and 1.87% of the students never experienced exam stress.

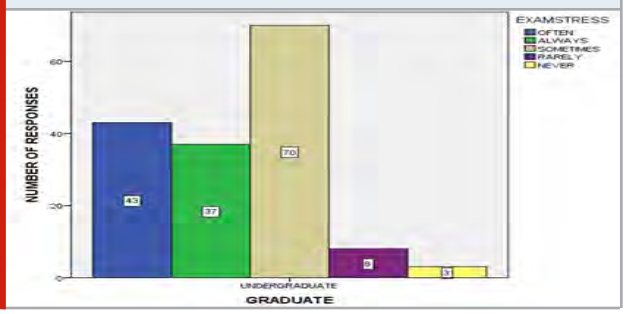
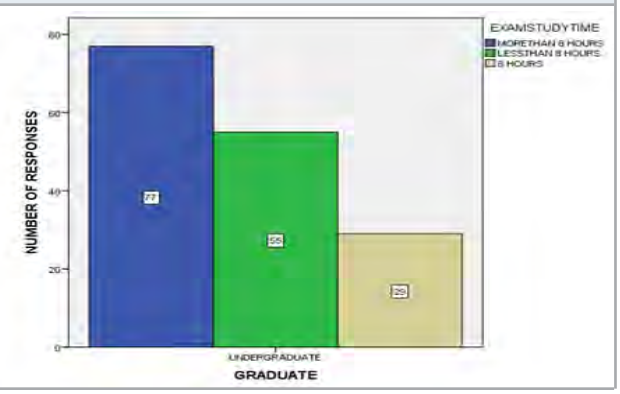


Figure 9: Bar graph shows the frequency distribution of students devoting their time to study during exam. It shows that 48.12% of the students study for more than 8 hours during the exam time, 34.7% of the students study for less than 8 hours during exam time and 18.2% of the students studied exactly 8 hours a day during exam time.



In figure 10 shows that 33.7% of the students (study population) have very often felt that they could have done better if they had devoted more time in studies, 24.37% of the students often felt the same, 32.5% of the students sometimes felt that they could have done better during exams, 1.87% of the students rarely felt and 1.87% of the students never felt that they could have done better even though they invested more time towards studies. In figure 13 we showed that 9.3% of the students spend more than 3 hours for their recreation, 40.6% of the students intermittently take breaks while studying and 50.65% of the students spend less than 3 hours for recreation. Around 39.3% of the students often spend time with their friends and family members in the study holidays, 26.25% of the students very often spend their time with them, 31.8% of the students sometimes spend time with friends and family and 3.12% of the students spend their time intermittently (figure 16).

Figure 10: Bar graph shows the frequency distribution of students afraid of getting grades lower than your friends. It shows that 17.5% of the students always had the fear of getting low grades than their friends, 52.5% of the students sometimes felt that they were afraid and 30.6% of the students never had the fear of getting low grades.

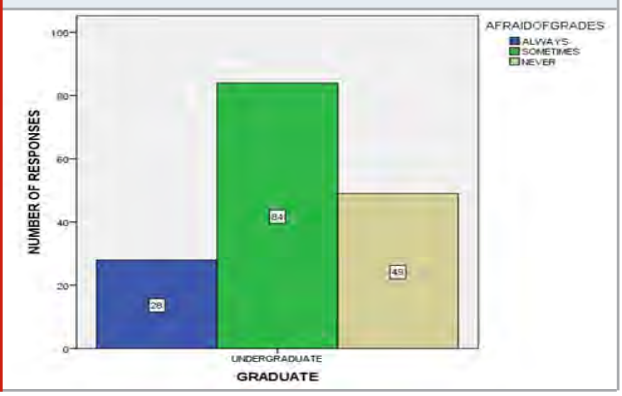


Figure 11: Bar graph shows the frequency distribution of students devoting their recreation time on a routine basis. It shows that 9.3% of the students spend more than 3 hours for their recreation, 40.6% of the students intermittently take breaks while studying and 50.625% of the students spend less than 3 hours for recreation.

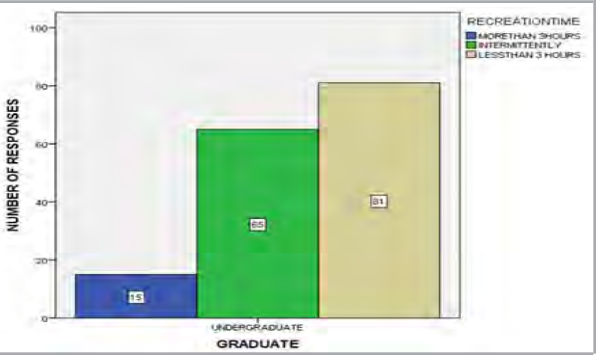
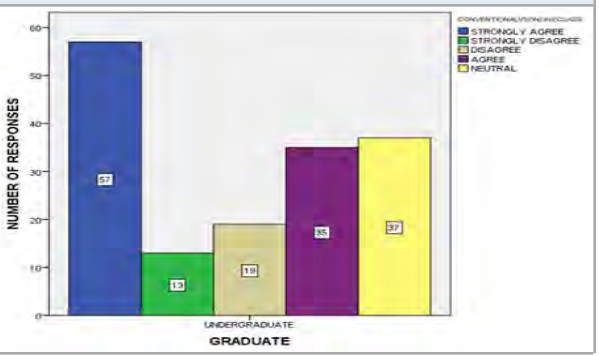


Figure 12: Bar graph shows the frequency distribution of students agreeing for conventional classroom learning rather than online learning. 35.6% of the students strongly agreed that classroom learning is more effective than the recent online learning, 8.12% of the students strongly disagreed that they did not felt that classroom learning did not prove to be very effective than the online learning, 11.8% of the students disagreed, 21.8% of the students agreed to the same and 23.12% were neutral regarding the statement.



As shown in the figure 17, 27.5% of the students strongly agreed that professional courses needed more time investment than other courses, 3.75% of the students strongly disagreed to the same, 6.875% of the students disagreed, 38.75% of the students agreed and 17.5% of the students felt neutral regarding the said statement. A study done by Classens et al. (2007) characterized time management as "behaviours that aim at achieving an effective use of time while performing certain goal-directed activities". Broadly, time management alludes to exercises that infer a successful utilization of time that is considered to encourage efficiency and ease pressure. A typical component among conceptualizations of time management is "planning behaviour (Classens et al., 2007). Divide and rule (divide et impera in Latin) is a policy discovered by British ruling system in India to gain and maintain large concentrations of power into pieces

of lesser power. This rule will be applicable to studies also in the matter that a larger part of the study area may be divided into small portions and then it will be easier for the reader to understand the concepts and retain the matter for a longer period (Sandhu, 2009).

Figure 13: Bar graph shows the frequency distribution of students spending their time with family, friends and others. It shows that 39.3% of the students often spend time, 26.25% of the students very often spend their time with them, 31.8% of the students sometimes spend time with friends and family and 3.12% of the students spend their time intermittently.

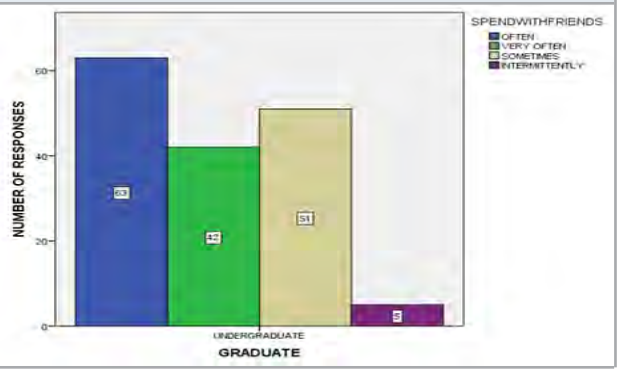
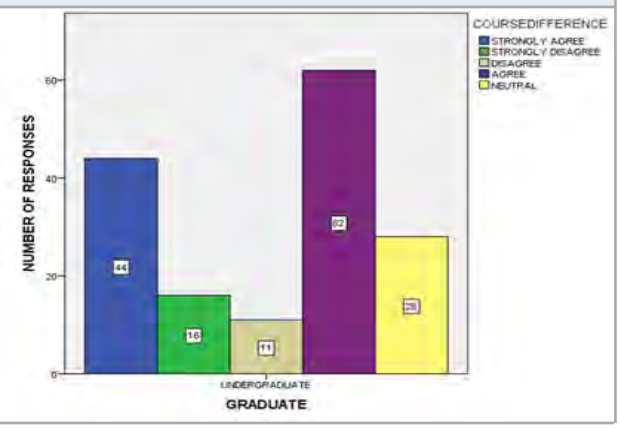


Figure 14: Bar graph shows the frequency distribution of students agreeing to differences in investing time for studies for professional courses than other courses. It shows that 27.5% of the students strongly agreed that professional courses needed more time investment than other courses, 3.75% of the students strongly disagreed to the same, 6.875% of the students disagreed, 38.75% of the students agreed and 17.5% of the students felt neutral regarding the said statement.



In our study, we included the same and obtained a result that 21.8% of the students strongly agreed to the statement 'Divide and Rule' worked out very well for them, 13.75% of the students disagreed with the statement and 64.3% of the students agreed with the statement (figure 11). As a usual phenomenon, the students compare the marks (Falchikov and Goldfinch, 2000) with the marks of their peers after the exam results. We included these criteria in our study and found that 17.5% of the students always had the fear of getting low grades than their friends, 52.5% of the students sometimes felt that they were afraid and 30.6% of the students never had the fear of getting low grades (figure 12).

Figure 15: Bar graph shows the association between gender and the recreation time during their daily routine. X-axis represents the gender and Y axis represents the number of responses to recreation time. Majority (40.6%) of the female students agreed that they spend less than 3 hours for their recreation time than male students. There is a significant difference between the group and recreation time. Chi square test was used to associate the variables, p value 0.000 ($p<0.05$), Hence statistical significance present.

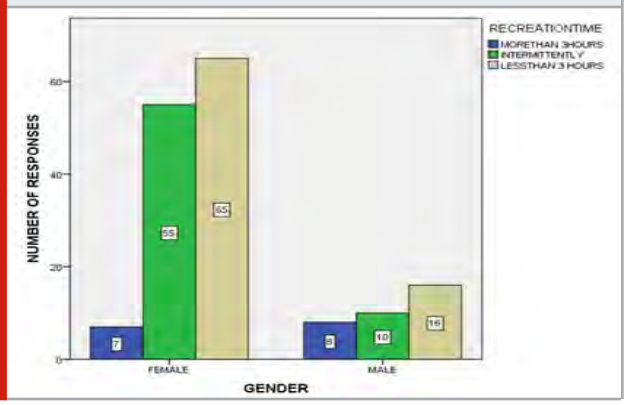
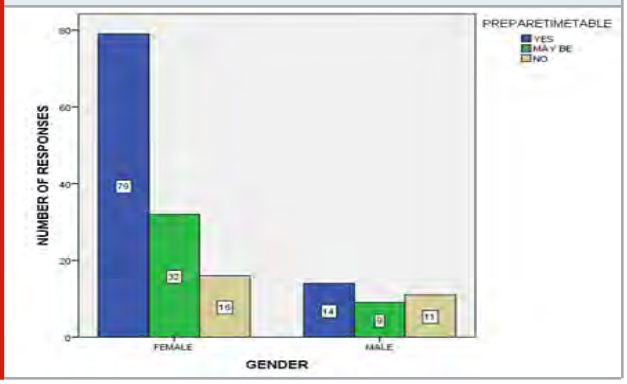


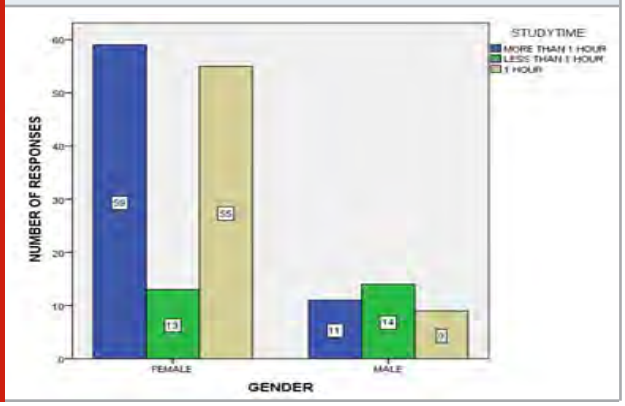
Figure 16: Bar graph shows the association between gender and the opinion on preparing timetables to study on a daily basis. X-axis represents the gender and Y axis represents the number of responses regarding the opinion on preparing timetables to study on a daily basis. Majority (49.37%) of the female students felt that preparing timetables for studying will therefore improve their quality time more than male students. There is a significant difference between the group and recreation time. Chi square test was used to associate the variables, p value 0.000 ($p<0.05$), Hence statistical significance present.



58.12% of the students agreed that preparing timetables would help them in studying systematically, 25.6% of students agreed that maybe the preparation of timetable could have been of some help and 16.8% of the students

disagreed with the statement (figure 18). A traditional and conventional classroom teaching and learning is now becoming an old entity as the students and the teaching systems are moving towards e-learning (Zhang et al.., 2004). In this belief, we found that 29.3% of the students often agreed that classroom learning could have been more creative, 27.5% of the students always felt that classroom learning could have been more creative, 51.25% of the students sometimes felt the same and 4.3% of the students rarely felt the need to incorporate creative thinking in classroom learning(figure 14).

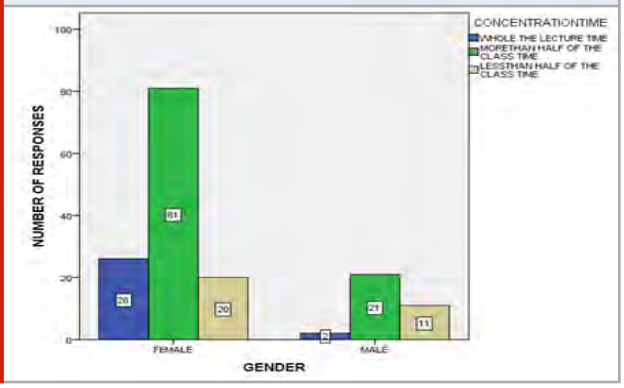
Figure 17: Bar graph shows the association between gender and study time. X-axis represents the gender and Y axis represents the number of responses regarding the study time on a daily basis. Majority (36.8%) of the female students spent more than 1 hour for their studies on a regular basis than male students. There is a significant difference between the group and recreation time. Chi square test was used to associate the variables, p value 0.000 (p<0.05), Hence statistical significance present.



Out of 100% study population, 35.6% of the students strongly agreed that classroom learning is more effective than the recent online learning, 8.12% of the students strongly disagreed that they did not feel that classroom learning did not prove to be very effective than the online learning, 11.8% of the students disagreed, 21.8% of the students agreed to the same and 23.12% were neutral regarding the statement(figure 15). In our study we compared the results among the male and female students and we found that a majority (40.6%) of the female students agreed that they spend less than 3 hours for their recreation time than male students($p<0.05$) (figure 19), majority (49.37%) of the female students felt that preparing timetable for studying will therefore improve their quality time than male students and the p value obtained was <0.05 (figure 20). Majority (36.8%) of the female students spent more than 1 hour for their studies on a regular basis than male students with a p value of <0.05 (figure 21) and incidentally we found that majority (50.6%) of the female students were able to concentrate for more than half the lecture time than male students(figure 22).

A logical reality demonstrates that a human cerebrum can focus for just 17 minutes in one go. Sitting before a PC or keeping the books open, while the brain is in a through and through various world, would have neither rhyme nor reason. At the point when surge starts with one undertaking then onto the next without taking any breaks, it is hard to value one's endeavours of sitting constantly in the work area. Lively walks, accomplishing something in between these times5 can be a useful distraction. Taking breaks while considering is worthy yet transforming examination time into unwinding time that won't be acknowledged.

Figure 18: Bar graph shows the association between gender and concentration time during a lecture class. X-axis represents the gender and Y axis represents the number of responses regarding concentration time during a lecture class. Majority (50.6%) of the female students were able to concentrate for more than half the lecture time than male students. There is a significant difference between the group and recreation time. Chi square test was used to associate the variables, p value 0.000 (p<0.05), Hence statistical significance present.



Envision how much time is spent to squander in the event of 4 hours on TV or Internet. Numerous examinations have demonstrated that there is an ideal condition between a sound way of life and work efficiency. Getting enough rest, practicing and eating good food helps vitality level and permits us to concentrate all the more without any problem. Innovation is acceptable. Utilization of a schedule application or schedule journal consistently can make a difference where it tracks the time and marks the important assignments assigned. It is a productive instrument for time management.

CONCLUSION

From this survey, it can be concluded that female students spend more time studying their daily portions than the male students. Time management has been a long time discussion topic and is still being discussed as new improvisation in the methods of learning have constituted too many solutions to help students overcome their exam stress and fear. This study gives a better understanding and knowledge about time management among various college students pursuing different courses. More papers can be submitted in order to help students overcome their exam fear and stress and also show light on how to manage time effectively

during virtual classes. There are certain limitations as to the equal proportion of participants from different courses.

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Conflict of Interests: Authors declare no conflict of interest.

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Molecular Studies of Antibiotic Resistant Genes in Some Gram-Positive Bacteria Isolated from King Fahad Hospital, Jeddah, Saudi Arabia

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ABSTRACT

The widespread and a dramatic increase usage of antibiotics in recent years led to appearance of new bacterial resistance to a various classes of antibiotics. The bacterial resistance to different antibiotics leads to increased morbidity and mortality associated with multidrug-resistant (MDR), particularly in pathogenic bacteria that infect people and cause infections. The current study was aimed to study and evaluate the resistance of some chosen Gram positive bacterial strains, it were *Bacillus cereus*, *Enterococcus faecalis, Staphylococcus aureus* and *Staphylococcus saprophyticus* isolated from King Fahad Hospital, Jeddah, Saudi Arabia to 16 different chosen antibiotics. The evaluation was done through disk diffusion assay method. Results concluded that the bacterial strains under study were resistant to all used antibiotics. This main point of study was confirmed by the presence of antibiotic resistance genes i.e. *ampR, blashv-12, penA1, penA2, ampC1, ampC2, aac4A-cr*, and *vanR* on plasmid and genomics DNA isolated from used bacterial strains. The obtained results highlights that the increase and rapid used of antibiotic resistance lead to appearance of MDR strains

KEY WORDS: ANTIBIOTIC RESISTANCE, PATHOGENIC BACTERIA, DNA, PLASMIDS, GENES.

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INTRODUCTION

Bacteria are leading causes for infectious diseases in humans. Gram-positive bacteria's are considered as main causative agents for animal intestinal and potentially lethal foodborne diseases in humans (Songer 2010; Maurya and Agarwal, 2019).One among the class of gram positive bacteria's are *Bacillus cereus* and is common in nature. This bacterium is isolated from

plants soil, and intestinal tracks of some mammals and insects. From these habitats, there are many sources by which it is spreader and are responsible for an emetic or a diarrhoeal type of food-related (Arnesen et al., 2008). Another important class of these bacteria's are *Enterococci* which are found in human faecal flora and are main causative agent for the Nasocomial infections in humans. thr. Human Enterococcal infections are caused by two species, *Enterococcus faecalis* and *Enterococcus faecium* (Lebreton et al., 2014).

Antimicrobial resistance is a growing public health threat worldwide and it is due to massive imprudent usage of antibiotics in clinical practice that is responsible for resistance in bacterial species. (Livermore, 2003; Tenover, 2006; Kapoor et al., 2017). There is a global concern about the appearance and growth of bacterial resistance to commonly used antibiotics (Cizman, 2003; Maurya and Agarwal, 2019). Some pathogenic bacteria developed resistance to multiple classes of antibiotics. The loss of effective antibiotics will undermine the management of the common infectious complications in susceptible patients undergoing chemotherapy for cancer, dialysis for renal failure and surgery, especially organ transplantation, for which the ability to treat secondary infections is crucial and even our ability to fight bacterial diseases (Tanwar et al., 2014).

There are various reasons that led to the spread of antibiotic resistance and possibly the most important cause is the non-application of infection control within the hospitals and outside. It has been recognized that methicillin-resistant *Staphylococcus aureus* (MRSA) in a hospital and in the community are genetically related. Another reason is the use of antibiotics in non-human outlets. A combination of microbial properties, the selective pressure of antibiotic use, communal and technical changes that enhance the transmission of resistant organisms has been attributed to the increase in bacterial resistance to antibiotics (Borer et al., 2002). Many conjugative transposons and plasmids, integrons and bacteriophages discovered to date encode antibiotic resistance proteins and can be transferred from donor bacteria to recipient bacteria via horizontal gene transfer. Resistance could be acquired also by a mutation in the genes in the. Resistance mechanisms probably have developed from genes present in organisms that produce antibiotics (Poole, 2002; Tanwar et al., 2014).

Further some small bacterial regulatory RNAs, categorized as RNA attenuators and small RNAs, coud control the expression of some resistance genes. For example, small RNA sprX, can formulate bacterial resistance to glycopeptide antibiotics through the specific downregulation of SpoVG protein. Modifications of the target enzymes or antibiotics can prevent the binding to targets, which act to confer high levels of resistance in respiratory/oral bacteria (Jiang et al., 2018). Resistant bacteria can be transmitted to humans through direct contact and via food chain or indirectly from the ecological pollution of farm effluents. Therefore, the current study was aimed to re-evaluate the antibiotic-resistance statues for some Gram-positive by the molecular characterization of these bacteria. Our PCR results from both genomics and plasmid DNA confirmed presence of resistant genes for different antibiotics in these bacterial strains.

MATERIAL AND METHODS

Bacterial Strains: Following Gram-positive were used for this study (*Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Staphylococcus saprophyticus*). And all the strains were kindly obtained from King Fahad Hospital, Jeddah, Saudi Arabia.

Antibiotic susceptibility testing rings: MASTRING-ST obtained from Mast Group Ltd. Bootle, Merseyside, U.K. an antibiotic susceptibility ring device for the convenient, simultaneous testing of the equivalent of six antibiotic discs as shown in supplementary Table (1).

Preparation of bacterial suspension: Using a sterile loop, three discrete colonies were taken from a 24 hour culture of the the bacteria and were inoculated into3 ml of nutrient broth. The broth was incubated for 2-3 hours until a slight visible turbidity appears. The growth of the bacteria was checked by the spectrophotometer at absorbance at 630 nm.

Performing the disk-diffusion susceptibility test: A sterile cotton swab was dipped into the standardized suspension, and excess broth was expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid level. To inoculate the agar plates, the swab was then streaked evenly in three directions over the entire surface of the Mueller-Hinton agar plate to obtain a uniform inoculum. A final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for 3 to 5 minutes.

The antibiotic impregnated discs were applied to the surface of the inoculated plate, using sterile forceps. All disks were gently pressed down into the agar with the forceps to ensure complete contact with the agar surface and incubated at 37 C for 20 hours as per Kiser et al., 2011. Primer design The sequences of antibiotic resistance genes were obtained from GeneBank nucleotide databases. The primers for each antibiotics resistant gene were designed by primer3 program The names of antibiotic resistant genes, their accession numbers, primer sequences and Sizes of RT-PCR Products as shown in Table (1).

Plasmid and genomic DNA Extraction Protocol: The plasmid and genomic DNA extraction was carried out by plasmid extraction mini prep kit and Thermo Scientific GeneJET Chromosomal Purification Kit respectively (Thermo Scientific GeneJET) according to according to manufacturer's instruction.

Polymerase chain reaction (PCR): All samples were placed in a polymerase chain reaction program, the PCR program was run as the follows, 40 cycles at 95°C

for 4 min, 95°C for 15 s, 50-60°C for 18 s, 72°C for 30 s and a final extension at 72°C for 7 minutes. The PCR products were analyzed on a 2% agarose gel stained with 5 µl Ethidium Bromide (EtBr). Bands on the gel were visualized in gel documentation system.

Figure 1: Disk-diffusion susceptibility test for the Gram-positive bacteria; Bacillus cereus (A), *Enterococcus faecalis* (B), *Staphylococcus aureus* (C) and *Staphylococcus saprophyticus* (D), using AK, CAZ, CIP, ATM, IMI and PRL antibiotics.

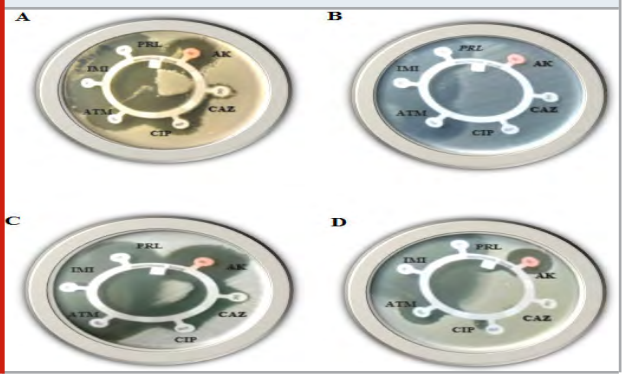
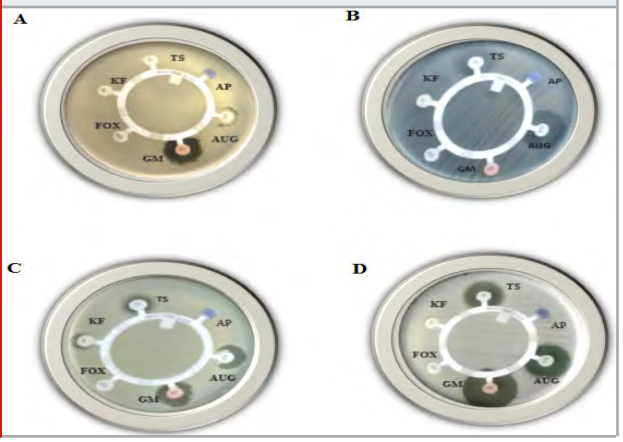


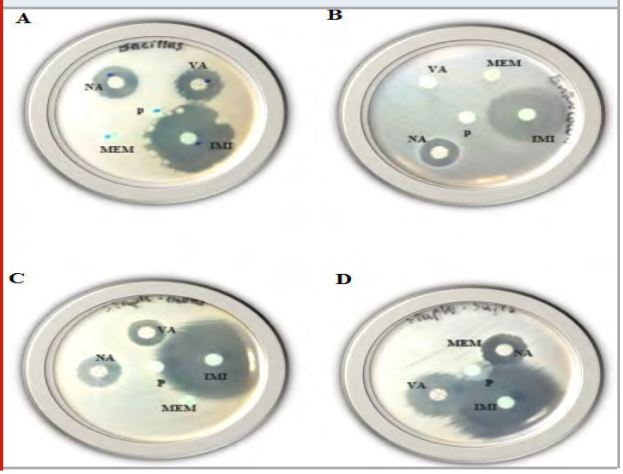
Figure 2: Disk-diffusion susceptibility test for the Gram-positive bacteria; *Bacillus cereus* (A), *Enterococcus faecalis* (B), *Staphylococcus aureus* (C) and *Staphylococcus saprophyticus* (D), using AP, AUG, GM, FOX, KF and TS antibiotics.



RESULTS AND DISCUSSION

Disk-diffusion susceptibility results: In the present study, the four mentioned Gram-positive species were examined for their resistance against 16 different antibiotics using disk-diffusion susceptibility test. The inhibition zone diameter chart was as follows; resistant: 13 mm or less, intermediate: 14-16 mm, sensitive: 17 mm or more. If present, the diameter of the inhibition zones in the current study ranged from 14 -77 mm.As shown (Table, 3) and Figures (1, 2 & 3) all mentioned species revealed resistance to *Ampicillin, Aztreonam, Ceftazidime, Meropenem and Penicillin*, while all of them found to be resistant to Augmentin and Cefoxitin antibiotics except Staphylococcus saprophytic, which showed sensitivity to both. As well as all of them were resistant to Cephalothin antibiotic except *Staphylococcus aureus*. *Bacillus cereus* and *Enterococcus faecalis* both were resistant to Sulfadiazine/trimethoprim antibiotic. On the other hand, three species showed resistance to only on antibiotic, *Staphylococcus aureus, Enterococcus faecalis* and *Enterococcus faecalis* found to be resistant to Piperacillin, Amikacin and Vancomycin antibiotics, respectively.

Figure 3: Disk-diffusion susceptibility test for the Gram-positive bacteria; *Bacillus cereus* (A), *Enterococcus faecalis* (B), *Staphylococcus aureus* (C) and *Staphylococcus saprophyticus* (D) using IMI, MEM, VA, NA and P antibiotics.



Localization of antibiotic resistance genes: All plasmids and genomic DNA isolated from the four mentioned species were separately tested by PCR to determine the presence of antibiotic resistance genes. *Bacillus cereus* samples were tested by PCR to determine the presence of antibiotic resistance genes (*ampR, blashv-12, penA1, ampC1, aac4A-cr, ampC2* and *penA2*). Plasmids found to harbor four out of the six tested antibiotic resistance genes (*blashv-12, ampC1, aac4A-cr and penA2*) with molecular sizes of 491, 323, 296 and 235 bp, respectively (Figure, 4). Chromosomal DNA harbored four out of the six tested antibiotic resistance genes (*ampR, blashv-12, ampC1 and penA2*) with molecular sizes of 310, 491, 323 and 235 bp, respectively (Figure, 5).

Enterococcus faecalis samples were tested by PCR to confirm the presence of *ampR, blashv-12, penA1, ampC1, aac4A-cr, ampC2, penA2* and *vanR*. Plasmids found to harbor four out of the eight tested genes *blashv-12, ampC1, aac4A-cr* and *ampC2*, with molecular sizes of 491, 323, 226 and 296 bp, respectively, as shown in Figure (6). Chromosomal DNA found to harbor four out of the eight tested antibiotic resistance genes (blashv-12, ampC1 and penA2) with molecular sizes of 491, 323 and 235 bp, respectively, as shown in Figure (7). *Staphylococcus aureus* samples were also tested by PCR to determine the presence of antibiotic resistance genes (*ampR, blashv-12, penA1, ampC1, ampC2* and *penA2*).

Plasmids harbored four out of the six tested antibiotic resistance genes (*blashv-12, ampC1, ampC2* and *penA2*) with molecular sizes of 491, 323, 296 and 235 bp, respectively, as shown in Figure (8). Chromosomal DNA found to harbor four out of the six tested antibiotic resistance genes (*ampR, blashv-12,* and *ampC1*) with molecular sizes of 310, 491, and 323 bp, respectively, as shown in Figure (9).

Table 3. Resistance and susceptibility patterns of some Gram-positive bacteria to some antibiotics using disk-diffusion susceptibility test

Antibiotic Name	Bacteria/Diameter of inhibition zone in mm			
	Bacillus cereus	*Enterococcus faecalis*	*Staphylococcus aureus*	*Staphylococcus saprophyticus*
Amikacin (AK)	20	R	40	23
Gentamicin (GM)	20	20	34	25
Ampicillin (AP)	R	R	R	R
Augmentin (AUG)	R	R	R	18
Aztreonam (ATM)	R	R	R	R
Cefoxitin (FOX)	R	R	R	R
Ceftazidime (CAZ)	R	R	R	R
Cephalothin (KF)	R	R	50	R
Ciprofloxacin (CIP)	20	74	22	16
Imipenem (IMI)	36	77	40	46
Meropenem (MEM)	R	R	R	R
Penicillin (P)	R	R	R	R
Piperacillin (PRL)	16	20	R	14
Trimethoprim/(TS Sulfamethoxazole	R	R	40	75
Vancomycin (VA)	22	R	10	32
Ampicillin (AP)	R	R	R	R
Nalidixic acid (NA) R: resistance	20	30	30	22

Figure 4: Detection of *Bacillus cereus* antibiotic resistance genes of DNA plasmids using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12; 3, penA1; 4, ampC1; 5, aac4A-cr; 6, ampC2; 7, penA2 and M refers to 50 bp DNA ladder.

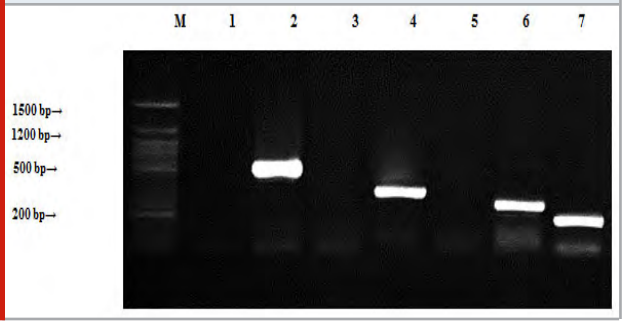
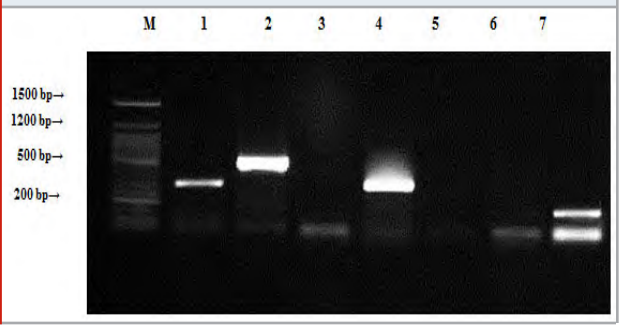


Figure 5: Detection of *Bacillus cereus* antibiotic resistance genes of chromosomal DNA using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12; 3, penA1; 4, ampC1; 5, aac4A-cr; 6, ampC2; 7, penA2 and M refers to 50 bp DNA ladder.



Staphylococcus saprophyticus samples were tested by PCR to decide the presence of genes *ampR, blashv-12, penA, ampC1, ampC2* and *penA2* genes. It found to harbor three out of the six tested antibiotic resistance genes (*ampR, blashv-12* and *aac4A-cr*) with molecular sizes of 310, 491 and 226 bp, respectively, as shown in Figure (10). Chromosomal DNA found to harbor four out of the six tested antibiotic resistance genes (*blashv-12, ampC1* and *penA2*) with molecular sizes of 491, 323 and 235 bp, respectively, as shown in Figure (11).

Gram-positive bacteria are the most common bacterial pathogens that cause diseases in humans, with streptococci and staphylococci occurring most frequently. Antibiotic resistance among Gram-positive bacteria has been increasing steadily during the past several decades.

(Turutoglu et al., 2006). In the current study, Bacillus cereus found to be sensitive to two out of the ten used β-lactam antibiotics (Imipenem and Piperacillin), while was resistant to eight of them. It also found to be sensitive to Aminoglycoside (Amikacin and Gentamicin), sensitive to Quinolones (Ciprofloxacin and Nalidixic acid), resistant to Trimethoprim/Sulfamethoxazole as member of Sulfonamides group and sensitive to Vancomycin from Glycopeptides group.

Figure 6: Detection of *Enterococcus faecalis* antibiotic resistance genes of plasmid DNA using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12; 3, penA1; 4, ampC1; 5, aac4A-cr; 6, ampC2; 7, penA2; 8, vanR and M refers to 50 bp DNA ladder.

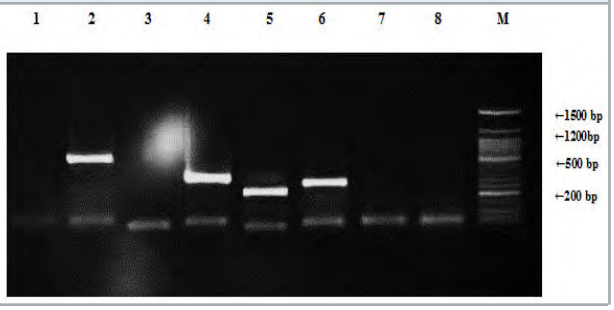


Figure 7: Detection of *Enterococcus faecalis* antibiotic resistance genes of chromosomal using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12; 3, penA1; 4, ampC1; 5, aac4A-cr; 6, ampC2; 7, penA2; 8, vanR and M refers to 50 bp DNA

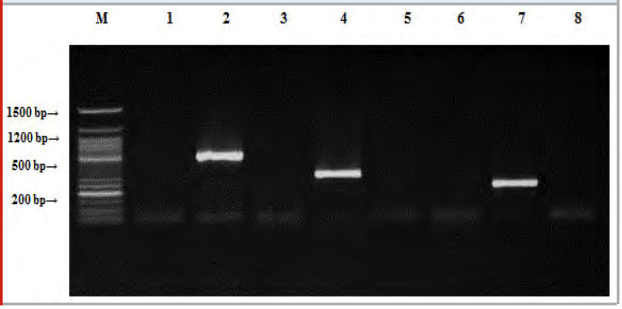


Figure 8: Detection of *Staphylococcus aureus* antibiotic esistance genes of DNA plasmids using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12, 3, penA1; 4, ampC1; 5, ampC2; 6, penA2 and M refers to 50 bp DNA ladder.

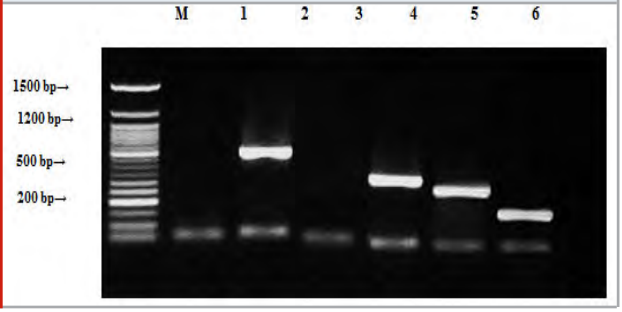


Figure 9: Detection of *Staphylococcus aureus* antibiotic resistance genes of chromosomal DNA using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12, 3, penA1; 4, ampC1; 5, ampC2; 6, penA2 and M refers to 50 bp DNA ladder.

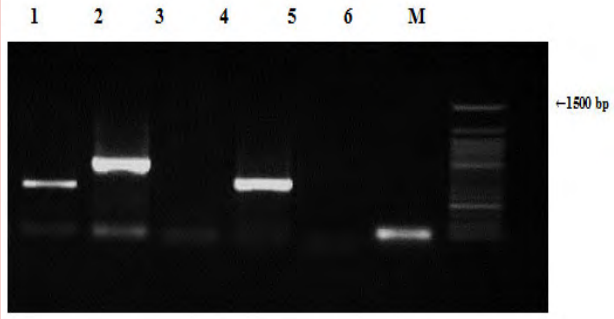


Figure 10: Detection of *Staphylococcus saprophyticus* antibiotic resistance genes of DNA plasmids using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12; 3, penA1; 4, ampC1; 5, ampC2; 6, penA2 and M refers to 50 bp DNA ladder.

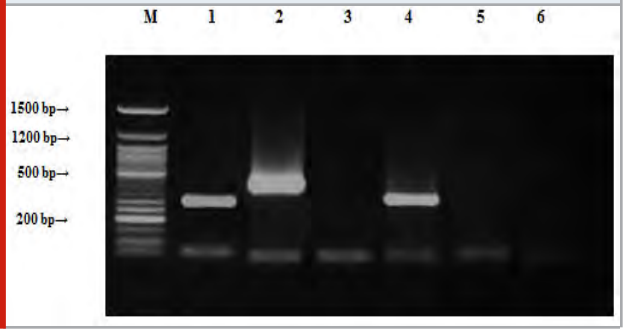
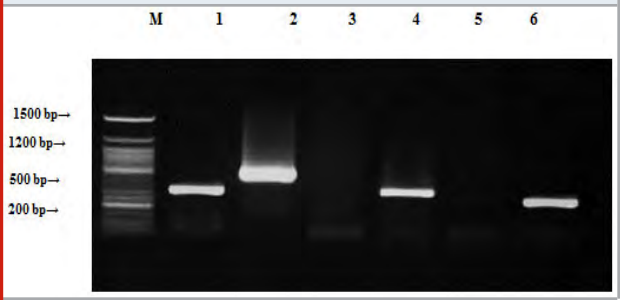


Figure 11: Detection of *Staphylococcus saprophyticus* antibiotic resistance genes of chromosomal DNA using agarose gel electrophoresis of the PCR products. Lane 1, ampR; 2, blashv-12; 3, penA1; 4, ampC1; 5, ampC2; 6, penA2 and M refers to 50 bp DNA ladder.



A previous study by Whitman et al. (1998) reported that, all *Bacillus cereus* strains was susceptible to Imipenem, Vancomycin, Gentamicin and Ciprofloxacin. They also reported that, *Bacillus cereus* was rarely susceptible to *Penicillin*. *Bacillus cereus* is usually sensitive to *Aminoglycosides* and *Vancomycin*. Part of our findings are in accordance with the findings of a previous study (Leach et al., 2007). On the other hand, in contrast to the

same mentioned previous study, our results revealed that, *Bacillus cereus* has acquired resistance to new antibiotics, which it was sensitive to before. This increased resistance might came from acquiring resistance genes from other bacterial species via horizontal gene transfer by time.

Our results showed that, *Enterococcus faecalis* was resistant to most of the tested β-lactam antibiotics except for Imipenem and Piperacillin. It also found to be resistant to Amikacin as an Aminoglycoside, Trimethoprim/Sulfamethoxazole (Sulfonamides) and Vancomycin (Glycopeptides). This is consistent with another study, which reported that, *Enterococcus faecalis* was resistant to Ampicillin, Penicillin, and Gentamicin, while the difference found is the resistance of *Enterococcus faecalis* to Ciprofloxacin (Mengeloglu et al., 2011).The present study results revealed *Staphylococcus aureus* as a resistant species to some β-lactams such as Ampicillin, Augmentin, Aztreonam, Cefoxitin, Ceftazidime, Meropenem, Penicillin and Piperacillin. This results are consistent with previous studies that proved *Staphylococcus aureus* is resistant to Penicillin, β-lactams and Cephalosporins (McDougal and Thornsberry, 1986; Turutoglu et al., 2006).

Staphylococcus saprophyticus resisted most of the tested β-lactam antibiotics (Ampicillin, Aztreonam, Ceftazidime, Cephalothin, Meropenem and Penicillin), these results are in accordance with the results of another study, which reported that, *Staphylococcus saprophytic* was resistant to most of β-lactam antibiotics (Ferreira et al., 2013). In the present study, none of our *Staphylococci* species was resistant to vancomycin. Vancomycin has long been considered as an antibiotic of last resort for MDR staphylococci infections (Koksal et al., 2009). On the other hand, vancomycin resistance has developed first in enterococci, in *Staphylococcus aureus* and coagulase-negative staphylococci (Boneca and Chiosis, 2003, Palazzo et al., 2005).

This situation has led *Staphylococci* to become a serious health problem that medical practitioners should be concerned about. The extensive use of glycopeptides in hospitals has been related to decreased susceptibility to these agents. Unfortunately, the therapy chance of MDR staphylococci infections is gradually decreasing. Molecular genetic analysis by PCR technique using plasmids and chromosomal DNA isolated from the four tested species revealed that, they harbor some but not all of the β-lactam resistant genes, which they are resistant to their corresponding antibiotics. This result is acceptable because as previously mentioned (Thenmozhi et al., 2014), the extended spectrum β-lactamases (ESBL) are enzymes produced by a variety of Gram-negative bacteria, which confer an increased resistance to commonly used antibiotics.

In conclusion, some species harbor some of the resistance genes on the plasmids only, some have them on their chromosomal DNA only and others harbor them on both plasmids and chromosomes. Together, these findings further supported by the facts that, horizontal gene transfer or the process of swapping genetic material between neighboring "contemporary" bacteria, is another means by which resistance can be acquired. Many of the antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors that transfer these genes to other members of the same bacterial species, as well as to bacteria in another genus or species. Horizontal gene transfer may occur via three main mechanisms: transformation, transduction or conjugation. Acquired resistance genes can be further incorporated into the recipient chromosome by recombination (Roberts, 2005, Von Wintersdorff et al., 2016).

Although antibiotic resistance occurs naturally, overuse and misuse of antibiotics in humans and animals is accelerating the process. Steps can be taken at all levels of society to reduce the impact and limit the spread of resistance, including the public, who can help by preventing infection through good hygiene and vaccination, only using antibiotics when prescribed by a certified health professional, taking the full course and never sharing or using leftover antibiotics. Therefore, the current study shows the importance of monitoring antibiotic intake and resistance trends of nosocomial infections, especially with infection control measures, to prevent the occurrence and blowout of multi-drug resistant bacteria within the hospitals and outside.

Finally, improved knowledge of molecular mechanisms controlling MDR should facilitate the development of novel therapies and will help develop deeper understanding of the microbial organism's pathobiology. With MDR bacteria on the rise, new drugs have to be developed to combat bacterial antibiotic resistance, such as innate defense regulators, reactive oxygen species and microbial volatile compounds. In addition, searching for new ways to treat bacterial infections such as bacteriophages, their effective enzymes against bacteria and medicinal plants, could help in competing this MDR threat.

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An Electromyographic Study to Evaluate the Effect of Age and Gender on Masseter Muscle Activities During Chewing of Different Textured Foods

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ABSTRACT

EMG is being used now days for describing various mechanical attributes of food for their texture analysis, During EMG the generated bioelectrical activity of the muscles are used for providing the real time information for the complete process of chewing. The objective of this study was to investigate the effect of gender and age on the bioelectrical activities of masseter muscles obtained during mastication of five different textured foods namely *jelly, cake, dhokla, rasgulla* and *paneer*. The electromyographic study was conducted on twelve healthy human subjects [4 females aged between 28 to 30 years (Group 1), 4 females aged between 18-20 years (Group 2), and 4 males aged between 18-20 years]. Eighteen electromyographic variables were obtained from each experimental session. The values of various masticatory parameters of Group 1 and Group 2 subjects were not significantly ($p \leq 0.05$) different. Also comparison of male with female electromyographic data, obtained during mastication, showed no significant ($p \leq 0.05$) difference except for inter burst duration per chew and early burst duration. The study indicated that the group for electromyographic data acquisition can be formulated from the subjects of both genders with age varying from 18 to 30 years.

KEY WORDS: ELECTROMYOGRAPHY, MASTICATION, TEXTURE, GENDER, AGE.

INTRODUCTION

Food texture and mouthfeel are the two important characteristics for consumers' food preference and acceptance (Guinard and Mazzucchelli, 1996). Texture depends on mechanical, geometrical and surface characteristics of foods which are perceived through various sense organs. It is an important factor which affects food eating behaviour (Chen, 2009). Social, cultural, physiological and psychological factors mainly govern the attributes of texture (Szczesniak and Khan, 1971; Kohyama, 2015). Gummy or slimy food with hard particles or lumps is generally considered as unacceptable. Learning about texture of food is a continuous process (Szczesniak, 2002). Texture assessment of food is based on various physical and sensory parameters and governed by surface response of food inside the mouth, by masseter muscles activities and by auditory means. Electromyography is a non-invasive and upcoming technique being used to investigate the textural perceptions of foods as accessed by masseter muscle activities of human subjects during chewing. The electromyography studies have been conducted on some regionally prevalent foods like *japonica* rice, kelpsnack,

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buckwheat noodles (Kohyama et al., 2000; Kohyama et al., 2010; Kohyama et al., 2016; Kohyama et al., 2018; Rustagi, 2020).

Also, various mastication parameters have been analyzed during chewing, by electromyography, for some continental foods like processed cheese, gummy candy, marshmallow, dried prunes, rice crackers, sponge cake, carrot and chewing gums, apples, meat, beef, idli and cookies (Duizer et al., 1996; Karkazis and Kossioni, 1997; Mioche and Martin, 1998; Karkazis and Kossioni, 1998; Mathoniere et al., 2000; Kohyama et al., 2005; Ioannides et al., 2009; Miyaoka et al., 2013; Dhillon et al., 2020a; Dhillon et al., 2020b). Gender and age are the two attributes which govern the development of suitable foods on the basis of physiological and psychological characteristics. Electromyography studies have been conducted to access the chewing behaviour of elderly and young subjects also by considering their dental status (Kohyama et al., 2002). Various studies have been conducted to find the relationship between oral physiologies like effect of salivary production rate (Affoo et al., 2015). During ageing body composition changes in terms of cell mass, cell fat etc. (Steen, 1988) and human subjects adapt to these changes during mastication process (Peyron et al., 2004). The chewing pattern of different textured foods varies from person to person (Wilkinson et al., 2000; Kohyama et al., 2003; Kohyama et al., 2005).

In old age weak muscle action, swallowing problem (dysphasia), difficulty in chewing, poor dentition and incomplete bolus formation occurs (Kohyama et al., 2015). Age and gender effects were studied on the basis of different anatomical characteristics of human body like height, weight, size of face and also on the basis of orofacial strength (Zhuang et al., 2010; Clark and Solomon, 2012). During chewing male and female differences were studied on the basis of mean bite force, salivary rate and mandibular movement (Braun et al., 1996; Nagasawa et al., 1997; Inoue et al., 2006). Significant differences in the chewing patterns were observed, as analyzed through electromyography, between two wider age groups having a mean age difference of about 38 years (Kohyama et al., 2002). However, to the best of our knowledge no study has been conducted on the narrower age groups. Thus, the aim of this study was to find the effect of gender and age (a smaller age gap of maximum up to 12 years for relatively younger age groups) on the masseter muscle activities during chewing, through electromyography, of five different textured foods (Kohyama et al., 2015).

MATERIAL AND METHODS

For the subjects, the experimental setup of this experiment was approved by Ethical Committee of Guru Nanak Dev University, Amritsar, Punjab, India. Twelve volunteers, eight females divided in to two groups: Group I (aged between 18 to 20 years) and Group II (aged between 28-30 years) and four males aged between 18-20 years, free from any mastication problems were selected as subjects for this study (Kohyama et al., 2003). All subjects gave their written consent to participate in the study. For the electromyography (EMG), bioelectrical activities of both left and right masseter muscles were recorded, simultaneously, using EMG setup (MP-150 System, Biopac Systems Inc., Goleta, CA, USA). Five different textured foods namely jelly, cake, dhokla, rasgulla and paneer were selected for the study. Cake, dhokla and paneer were cut into pieces weighing 5 grams each while jelly and rasgulla (approx. 5 grams) were given in their original shape to the human subjects.

Bipolar surface electrodes (EL 503) were used to acquire EMG signals of masseter muscles from both sides of face which were then filtered (10-500Hz) with removal of noise at 50 Hz caused by the power supply and amplified (×1000) with EMG 100C amplifiers (Biopac Systems Inc.) and stored on PC at 1000 Hz frequency using MP-150 system (Biopac Systems Inc.) (Kohyama et al., 2014). EMG signals were acquired and analyzed using AcqKnowledge Software (ver. 4.4, Biopac Systems Inc.). The detailed procedure for setting up of electrodes on human subject, data acquisition and analysis were performed as discussed earlier (Rustagi et al., 2018a; Rustagi et al., 2018b). For the statistical analysis, the masseter muscle activities acquired using surface EMG were subjected to analysis of variance (ANOVA) test using Minitab Statistical Software (Minitab Inc., USA).

RESULT AND DISCUSSION

The acquired EMG data for both male and female subjects were analyzed from electromyogram to obtain eighteen EMG variables divided in to entire mastication period, per chew mastication and different stages of mastication viz. early, middle and late (Pratiksha et al., 2018; Sodhi et al., 2019). The mean values of duplicate EMG variables for both male and female subjects, while masticating five different textured foods, were subjected to analysis of variance. For the effect of age, the analysis of EMG variables obtained for Group I of female subjects (Table 1) revealed that EMG variables except muscle activity per chew, cycle time per chew, burst duration per chew and burst durations at different stages of mastication can differentiate ($p < 0.05$) these different textured foods. The cycle time per chew can also be used to distinguish these foods by lowering the level of confidence ($p < 0.10$).

So, it may be summed up here that out of eighteen EMG variables acquired to examine the bioelectrical activity of masseter muscles of female subjects, thirteen variables are effective ($p < 0.10$) in representing the textural differences of these five different textured foods investigated in the present study. The analysis of EMG variables obtained for Group II of female subjects (Table 2) revealed that EMG variables except muscle activity per chew and muscle activity at different stages of mastication can differentiate ($p < 0.05$) these different textured foods. A statistical analysis for comparison of EMG variables obtained from masseter muscles' activities by different aged subjects (Group I and Group II), while masticating five different textured foods, is presented

in Table 3. It was observed that during chewing there is no significant difference ($p < 0.05$) in the acquired EMG variables. This reveals that the chewing pattern of both age groups is similar. The significantly different values of masticatory parameters for young (mean age of 29.4 years) and elderly (mean age of 67.7 years) subjects were earlier reported (Kohyama et al., 2002; Matsuo et al., 2020).

Table 1. Electromyographic variables acquired from female subjects (Group I) during mastication of different textured foods.

EMG variables	Jelly	Cake	Dhokla	Rasgulla	Paneer	p-value
Chews	15.38	24.38	16.13	21.25	26.38	0.00
Mastication time (s)	13.34	19.96	13.00	16.49	20.17	0.02
Total burst duration (s)	4.67	7.73	5.06	6.64	8.12	0.00
Total muscle activity (mV·s)	0.26	0.54	0.33	0.69	0.54	0.01
Burst duration per chew (s)	0.30	0.31	0.31	0.31	0.31	0.97
Interburst duration per chew (s)	0.59	0.53	0.52	0.47	0.47	0.02
Cycle time per chew(s)	0.89	0.84	0.83	0.79	0.78	0.07
Muscle activity per chew(mV·s)	0.06	0.24	0.22	0.13	0.21	0.51
Amplitude per chew(mV)	0.91	1.00	0.84	1.21	0.89	0.01
Early burst duration (s)	0.21	0.16	0.10	0.15	0.09	0.23
Middle burst duration (s)	0.23	0.14	0.13	0.13	0.10	0.16
Late Burst duration (s)	0.19	0.15	0.10	0.14	0.12	0.51
Early muscle Activity (mV·s)	0.02	0.03	0.02	0.04	0.02	0.00
Middle muscle Activity (mV·s)	0.02	0.02	0.02	0.03	0.02	0.00
Late muscle Activity (mV·s)	0.02	0.02	0.02	0.03	0.02	0.00
Early amplitude (mV)	0.91	1.06	0.73	1.25	0.86	0.01
Middle amplitude (mV)	0.95	1.01	0.81	1.29	0.83	0.00
Late amplitude (mV)	0.90	0.97	0.94	1.21	0.88	0.01

Table 2. Electromyographic variables acquired from female subjects (Group II) during mastication of different textured foods.

EMG variables	Jelly	Cake	Dhokla	Rasgulla	Paneer	p-value
Chews	18.25	22.00	22.13	20.50	34.13	0.00
Mastication time (s)	14.74	19.32	17.58	17.25	25.44	0.00
Total burst duration (s)	4.76	5.79	5.47	5.97	8.50	0.00
Total muscle activity (mV·s)	0.45	0.58	0.52	0.71	0.82	0.00
Burst duration per chew (s)	0.28	0.28	0.26	0.29	0.26	0.00
Interburst duration per chew (s)	0.59	0.64	0.58	0.57	0.53	0.00
Cycle time per chew(s)	0.86	0.92	0.84	0.86	0.78	0.00
Muscle activity per chew(mV·s)	0.41	0.13	0.13	0.47	0.12	0.14
Amplitude per chew(mV)	1.15	1.23	1.12	1.37	1.15	0.00
Early burst duration (s)	0.23	0.22	0.18	0.17	0.20	0.00
Middle burst duration (s)	0.17	0.10	0.18	0.19	0.19	0.00
Late Burst duration (s)	0.20	0.10	0.16	0.14	0.14	0.00
Early muscle Activity (mV·s)	0.02	0.03	0.02	0.27	0.02	0.34
Middle muscle Activity (mV·s)	0.02	0.02	0.03	0.42	0.03	0.32
Late muscle Activity (mV·s)	0.03	0.03	0.02	0.27	0.02	0.34
Early amplitude (mV)	1.04	1.23	1.11	1.30	1.25	0.00
Middle amplitude (mV)	1.08	1.29	1.19	1.42	1.21	0.00
Late amplitude (mV)	1.26	1.17	1.07	1.37	1.04	0.00

Table 3. Comparison of Group I and Group II female subjects' electromyographic variables acquired during mastication of different textured foods.

EMG variables	p-values				
	Jelly	Cake	Dhokla	Rasgulla	Paneer
Chews	0.09	0.14	0.26	0.86	0.39
Mastication time	0.09	0.22	0.45	0.91	0.53
Total burst duration	0.22	0.32	0.58	0.88	0.5
Total muscle activity	0.76	0.85	0.8	0.84	0.8
Burst duration per chew	0.87	0.8	0.78	0.85	0.93
Interburst duration per chew	0.48	0.07	0.13	0.68	0.29
Cycle time per chew	0.5	0.14	0.1	0.61	0.21
Muscle activity per chew	0.34	0.79	0.81	0.17	0.82
Amplitude per chew	0.78	0.76	0.77	0.7	0.8
Early burst duration	0.32	0.51	0.73	0.25	0.71
Middle burst duration	0.69	0.29	0.58	0.52	0.78
Late Burst duration	0.64	0.11	0.71	0.37	0.66
Early muscle Activity	0.79	0.66	0.77	0.52	0.67
Middle muscle Activity	0.81	0.8	0.74	0.5	0.71
Late muscle Activity	0.68	0.71	0.79	0.51	0.77
Early amplitude	0.83	0.72	0.73	0.8	0.68
Middle amplitude	0.8	0.76	0.8	0.77	0.69
Late amplitude	0.71	0.73	0.8	0.65	0.82

Variations in preferences to different textures for younger (mean age of 32-33 years) and elderly (mean age of 75-76 years) subjects were also reported (Roininen et al., 2003). A study on subjects varying in age from 20 to 90 years and observed lesser amplitude for elderly subjects as with ageing there is change in the density and area of masseter muscles as well as decrease in number of teeth (Newton et al., 2003). A conclusion from the above discussion can be made that the chewing pattern is relatively similar for subjects having narrow age difference, among younger group of subjects aged between 18-30 years, as it was a maximum of 12 years in our study. This could be attributed to the fact that masseter muscle activities are more dependent on dental status, weakness of masticatory muscles with age, chewing and swallowing. There is also increase in mastication load which thus increases the muscle activity in elder subjects' disorders (Kohyama et al., 2003; Peyron et al., 2004; Kohyama et al., 2015).

It is highly unlikely that any such problem onsets in the relatively young age groups selected in this study irrespective of the age differences for both the groups (Matsuo et al., 2020). To study the effect of gender, significant differences ($p < 0.05$) were observed for the most of the EMG variables obtained for male subjects except mastication time, interburst duration per chew, cycle time per chew and burst durations at different stages of mastication for these five different textured foods (Table 4). However, out of these parameters mastication time can also be used to differentiate these foods at the reduced level of confidence ($p < 0.10$). So it may be concluded from this discussion that thirteen EMG variables obtained from the analysis of bioelectrical activities of masseter muscles of male subjects can effectively ($p < 0.10$) distinguish these five different textured foods investigated in the present study (Matsuo et al., 2020).

A statistical analysis to compare EMG variables obtained for masseter muscles' activities of male and Group 1 female subjects while masticating five different textured foods, is presented in Table 5. It was observed that there is no significant difference ($p < 0.05$) in the acquired EMG variables except for inter burst durations in case of jelly, cake and dhokla while early burst durations for cake, dhokla and rasgulla for male and female subjects. The inter burst durations were significantly ($p \leq 0.05$) higher for female subjects for jelly, cake and dhokla. These results may be attributed to the reason that female subjects require a greater number of chews and mastication time as they chew food slowly with low value of mastication force and exhibits longer bursts of muscle activity and total duration of cycle (Nagasawa et al., 1997; Youssef et al., 1997; Matsuo et al., 2020).

However, the early burst durations were higher in male subjects for cake, dhokla and rasgulla. These differences may be due to the reason that the frequency of mastication for both hard and soft foods is greater in male subjects (Khamnei et al., 2016). The differences in basal metabolic rate for male and female subjects may also be a contributory factor for these variations. According to differences in age and gender, oral

processing behaviour of consumers shows variations in their bolus properties however there is small differences observed during dynamic texture perception. The present study revealed that masseter muscle activities during mastication process of these five different textured foods, as a whole, are not affected by gender variations (Henry et al., 2018; Monica et al., 2020).

Table 4. Electromyographic variables acquired from male subjects during mastication of different textured foods.

EMG variables	Jelly	Cake	Dhokla	Rasgulla	Paneer	p-value
Chews	21.63	23.75	20.38	20.25	25.25	0.03
Mastication time (s)	15.78	18.82	14.81	16.06	18.41	0.08
Total burst duration (s)	5.85	7.34	5.79	6.52	7.27	0.05
Total muscle activity (mV·s)	0.35	0.47	0.37	0.46	0.38	0.04
Burst duration per chew (s)	0.27	0.31	0.28	0.32	0.28	0.00
Interburst duration per chew (s)	0.46	0.48	0.43	0.46	0.44	0.60
Cycle time per chew(s)	0.73	0.79	0.71	0.78	0.72	0.76
Muscle activity per chew(mV·s)	0.02	0.02	0.02	0.02	0.01	0.00
Amplitude per chew(mV)	0.77	0.81	0.79	0.88	0.62	0.01
Early burst duration (s)	0.33	0.36	0.34	0.42	0.36	0.37
Middle burst duration (s)	0.28	0.31	0.29	0.31	0.29	0.40
Late Burst duration (s)	0.26	0.27	0.26	0.28	0.23	0.41
Early muscle Activity (mV-s)	0.02	0.02	0.02	0.03	0.02	0.02
Middle muscle Activity (mV-s)	0.02	0.02	0.02	0.02	0.01	0.00
Late muscle Activity (mV-s)	0.02	0.02	0.02	0.02	0.01	0.02
Early amplitude (mV)	0.74	0.86	0.84	0.87	0.55	0.02
Middle amplitude (mV)	0.83	0.85	0.82	0.90	0.61	0.00
Late amplitude (mV)	0.80	0.85	0.80	0.90	0.55	0.03

Table 5. Comparison of male and female (Group I) subjects' electromyographic variables acquired during mastication of different textured foods.

EMG variables	p-values				
	Jelly	Cake	Dhokla	Rasgulla	Paneer
Chews	0.27	0.93	0.54	0.85	0.87
Mastication time	0.45	0.82	0.76	0.92	0.70
Total burst duration	0.44	0.87	0.78	0.95	0.76
Total muscle activity	0.64	0.80	0.88	0.55	0.61
Burst duration per chew	0.42	0.66	0.54	0.74	0.45
Interburst duration per chew	0.02	0.03	0.05	0.12	0.08
Cycle time per chew	0.24	0.52	0.39	0.94	0.54
Muscle activity per chew	0.31	0.22	0.22	0.34	0.19
Amplitude per chew	0.80	0.69	0.91	0.51	0.55
Early burst duration	0.20	0.05	0.02	0.03	0.07
Middle burst duration	0.63	0.14	0.10	0.11	0.07
Late Burst duration	0.49	0.17	0.16	0.19	0.36
Early muscle Activity	0.99	0.55	0.91	0.59	0.61
Middle muscle Activity	0.89	0.71	0.88	0.58	0.60
Late muscle Activity	0.71	0.57	0.65	0.32	0.33
Early amplitude	0.79	0.66	0.78	0.46	0.44
Middle amplitude	0.84	0.76	0.97	0.47	0.61
Late amplitude	**0.86**	**0.82**	**0.78**	**0.46**	**0.46**

CONCLUSION

The present study was conducted to acquire the masseter muscle activities of human subjects by electromyography during chewing of five different textured foods namely jelly, cake, dhokla, rasgulla and paneer. The study was undertaken to evaluate the effect of age difference and gender on the masseter muscle activities. No significant differences were found in various masticatory parameters as observed by electromyographic analysis for the selected age groups viz. 18-20 years and 28-30 years indicating that this narrow difference in age does not influence the chewing patterns. The analysis of acquired EMG variables also revealed that both male and female subjects can effectively ($p<0.10$) distinguished these different textured foods. Further on comparison of these EMG variables for male and female subjects, it was found that their mastication process is also similar. So it may be concluded that for EMG sessions mixed group of young male and female subjects having narrower age differences can be selected for design and development of different textured foods.

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The Effectiveness of Self-Administered Contract Relax Agonist Contract (CRAC) Stretching on Performance of Hamstring Curls to Fatigue in Elderly Population

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ABSTRACT

Active lifestyle is important for all age groups, especially essential among older adults to counter balance the deleterious effects of aging. Scientific literature is scarce about the effect of Proprioceptive Neuromuscular Facilitatio (PNF) stretching on older adults. Therefore, the purpose of this study is to find out the effect of self-administered CRAC stretching on the performance of hamstring curls to fatigue in older adults. We used the same subject repeated measure experimental crossover design, where subjects were randomly allocated into two groups and both the groups were given the Self-Administered CRAC stretching in alternate session to overcome the order effect. The result of this present study demonstrated that there was a significant between-group effect (t = -2.06, p=0.0484) seen. The participants getting CRAC stretching performed 37% and 44% less hamstring curls to fatigue in the respective sessions. In summary, our primary findings indicated enhanced flexibility with an accompanying decrease in the number of hamstring curls performance in response to self-administered CRAC stretching. These findings are unique in that, to our knowledge, no other authors have examined the effects of CRAC stretching on isolated muscle performance with a repeated measures design. Regarding the mechanisms underlying the stretching-induced performance deficit, the decreases in no of hamstring curls we observed in our study tentatively support the hypothesis that stretching may alter the length-tension relationship and the hypothesis that stretching may reduce muscle activation, respectively. CRAC stretching can be made part of a full warm-up routine because of its positive impact on flexibility and musculotendinous injury occurrence in the physically active older population.

KEY WORDS: CRACS-CONTRACT RELAX AGONIST CONTRACT, N-NUMBER, NHC- NUMBERS OF HAMSTRING CURLS, S1-SESSION 1, AND S2-SESSION 2.

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INTRODUCTION

Regular physical activities and exercise provide protection against many cardiovascular and lifestyle associated diseases, improve flexibility, and enhance the overall performance as well. Despite the rewards that physical activity and exercise bring, for the aging population, the compliance with this activity is quite low (Witvrouw et al 2004, Boulton et. al. 2018, Dazau et al 2019).

The age-related decrease in strength and flexibility negatively affects the activity level and exercise in the older population (Behm and Chaouachi, 2011 Behm et al 2015, Musich et al 2017 and Sato et al 2020). Due to decreased strength, flexibility, and comorbidities the physical activity and exercise carry the risk for injuries leading to disability in the older population (Rae et al 2020 Angulo et al 2020).

Studies comparing the effect of different stretching techniques have found to be effective in improving joint flexibility (Behm et al 2011). Samson et al (2012) demonstrated that, in comparison to dynamic stretching, passive stretching is more effective in improving the joint range of motion at rest. Proprioceptive Neuromuscular Facilitation (PNF) stretching has been reported to be superior to passive stretching in improving flexibility (Caplan et al 2009). Proprioceptive Neuromuscular Facilitation (PNF) techniques utilizing a specific sequence of passive stretching and isometric contractions provide a distinct improvement in muscle flexibility and power(Sharman et al 2006 Dzau et al 2019). PNF techniques require assistance, thereby making it difficult to perform. Two of the most used PNF techniques are the contract-relax (CR) and the contract-relax-antagonist-contract, popularly called as CRAC, (Dent et al 2019).

Maddigan et al (2012) have demonstrated that unaided PNF stretching with the help of a strap is equally effective in the normal population. Later, Burgess et al (2019) have shown that CRAC is a safe and effective method of improving hamstring flexibility. Even though the literature is scarce about the effect of PNF stretching on older adults it is often used to improving flexibility associated with physical activity and exercises. Given the contentious nature of research that has assessed the link between hamstring performance, flexibility, and injury. In addition to this, till date, no study has assessed the impact of self-administered CRAC stretching on the performance of hamstring curls to fatigue in the older adults. Therefore, the purpose of this study is to determine the effect of self-administered CRAC stretching on the performance of hamstring curls to fatigue in the older adults.

MATERIAL AND METHODS

Active older adults aged between 50 to 70 years, with no restriction in hip, knee, and ankle joint with grade 5 muscle strength on manual muscle testing scale, were included in the study. Participants having any musculoskeletal injuries, neurologic, cardiovascular, or any other disease affecting lower-limb, or any sensory-motor deficit were excluded from the study. Participants with cognitive deficit or non-compliant participants were excluded from the study. Informed written consent was taken from the subjects after explaining the risk factors, purpose, and procedure of the study, and participants were informed that they had the right to withdraw from the study at any stage. The same subjects were repeated with measure experimental crossover design. The participants were randomly allocated into two groups by lottery method and both the groups were given the Self-Administered CRAC stretching in alternate sessions to overcome the order effect. To achieve this in the first session Self-Administered CRAC stretching was given to the first group and in the second session subjects from the second group got self-administered CRAC stretching. The outcome variable was the maximum number of hamstring curls performed after the stretching. All the measurement was collected during a single session. Both the groups had fifteen subjects and were matched for age, sex, and body mass, to get a uniform sample.

Participants' maximal voluntary isometric contraction was established by strain gauge method before any intervention (Dara et. al. 2007). The subject was rested for 10 minutes and then performed submaximal warm-up exercises on the cycle ergometer. After the warmup, the subjects from the experimental group performed Self-Administered CRAC Stretching based on the protocol given by Maddigan et. al. (2012). The stretching was performed with the subjects lying supine on the couch with the contralateral leg secured to the couch with a strap placed over the upper thigh. The participants raised their legs straight up with a belt placed around the sole. Holding on both the ends of the belt the leg was pulled towards the torso until a mild stretch is felt in the hamstring. This position was maintained for seven seconds then the participants, isometrically contracted the hamstring and held it for seven seconds by attempting to push the leg back toward the table against the resistance of the belt by holding it tight and not letting the leg move. Maintaining this position, they relaxed the muscle for five seconds and stretched it again for seven seconds. The sequence they followed was 7 seconds of stretch, 5 seconds of isometric contraction, 5 seconds relax, and finally, seven seconds of stretch again, which was repeated five times on each subject. Subsequently, they performed as many hamstring curls as they can at a load of 70% of maximal voluntary isometric contraction, (Maddigan et al 2012, Huygaerts et al 2020).

The participants in the control group did not perform stretching instead after the rest period they performed as many hamstring curls as they can at 70% of maximal voluntary isometric contraction load. After 5-7 days of the gap, readings for the second session were collected while reversing the order of giving the Self-Administered CRAC stretching to the second group and then measuring the number of hamstring curls to fatigue. The first group performed hamstring curls to fatigue without Self-Administered CRAC stretching. The maximum numbers of hamstring curl were noted for both groups and in both test sessions.

RESULTS AND DISCUSSION

All the data were tabulated and analyzed using SPSS version 20. Demographic data of subjects including sex, age, height, and weight were descriptively summarized. To find the Self-Administered CRAC stretching on hamstring curls to fatigue performance, t-test was used keeping

the value of α=<0.05. In this study, we have taken 30 subjects with a mean age of 59.3+3.1 ranging from 50 to 68 years, average weight of 64.1+6.2 kilograms, and with a mean height of 164.1+4.9 centimeters. Required statistical test were performed to find out the effect of the experiment on the dependent variables, these findings are mentioned below. In the first session the participants who received CRAC stretching (M = 8.5, SD = 1.9) compared to the participants in the control group (M = 10.7, SD = 1.8) demonstrated significantly more hamstring curls to fatigue, t = 3.412, p = 0.002. A similar pattern was observed in the second session as well, where the number hamstring curls to fatigue, performed by the subjects getting CRAC stretching were less (M = 10.2, SD = 2) compared to the participants of control group (M = 12.9, SD = 2.4), which was a statistically significant t =3.356, p = 0.002 (Table 1).

A reduction of 21% and 20% hamstring curls were observed in the participants getting CRAC stretching respectively, in the first and second session. The results from the first session (M = 8.5, SD = 1.9) and second session (M = 10.2, SD = 2) on the performance of hamstring curls to fatigue showed an improvement, t = 2.229, p = 0.043 in the participants getting CRAC stretching (Table 1).There was a significant increase in the number of hamstring curls to fatigue performed by the participants in the control group between the first (M = 10.7, SD = 1.8) and second session (M = 12.7, SD = 2.4), t = 3.090, p = 0.008 (Table 1).

Table 1. Effect of CRAC stretching on hamstring curls to fatigue during two different sessions

	CRAC Stretching N=15		Control N=15		t-test	
	Mean	Std. Deviation	Mean	Std. Deviation	t	Sig. (2-tailed)
NHC_S1	8.5	1.9	10.7	1.8	3.412	.002
NHC_S2	10.2	2.0	12.7	2.4	3.156	.004
Paired t-Test	T	2.229	3.090			
	P	0.043	0.008			

In our study, we have used a self-administered contract-relax-antagonist-contract (CRAC) stretching technique. A single session of CRAC stretching has demonstrated a reduction in performance during hamstring curls to fatigue in the subjects. The result of our study is consistent with previous reports of a transitory decline in muscle performance after stretching. Behm et. al. (2015) have reported that the decline in muscle performance is mainly due to a decrease in motor unit activity and suggested it to be protective in nature as it prevents extreme fatigue of the muscle fiber by decreasing the frequency of the action potential. In a similar study, Davis et al. (2005) showed improved flexibility after a contract-relax agonist PNF stretching protocol but it was not found to be better than static or active-controlled stretching. However, the most likely factor for the difference between our studies is the fact that our participants belonged to the older adults, which might have affected their ability to be flexible. O'Hora et al. (2011) study also demonstrated a similar finding of enhanced flexibility due to agonist contract-relax PNF when compared with static stretching on hamstring length.

Recently, Caldwell et al. (2019) in their study, examined the effects of stretching on quadriceps muscle and showed a substantial reduction in the strength, but contrary to this, Palmer et al. (2019) observed a decline in muscle power only after stretching for longer duration. In another study Kay and Blazevich (2008), they demonstrated that sustained stretching of the muscle resulted in decreased muscle performance, but brief stretching does not adversely affect it. Another study by Reid et al. (2018) demonstrated an improvement in the knee flexibility and range of motion in response to stretching on the knee, they further elaborated that the stretching duration of ≤60 does not affect the maximum isometric power but with increased duration, there was a clear reduction in the muscle power.

The result of the study by Blazevich et al. (2018) was also similar as it showed no negative effect of short duration stretching exercised on muscle power. Bengtsson et al. (2018) examined the effect of sport-specific exercises on peak torque of the knee extensors and showed that stretching does not hamper muscle performance. Blazevich et al. (2018) also reported a beneficial effect of stretching on motivation due to the participant's belief that it can enhance their performance irrespective of type and duration of stretching. These findings contradict the widespread opinion that stretching inhibits performance and emphasizes the role of stretching duration on muscle power and performance (Pulverenti et al., 2019).

As our findings indicated as well as previous studies have shown that an acute bout of muscle stretching can improve flexibility but adversely affect the force output and performance. However, in a community or clinical setting, the mild to moderate decrease in muscle performance may not be relevant as the main objective of stretching in these settings are to achieve

a pain-free function for an active lifestyle. During the rehabilitation period, regaining functional range of motion is more important than maximal muscle-force production. Therefore, it is safe to recommend the use of short duration stretching as an integral part of the pre-exercise warm-up routine due to its positive psychological effect and its potential to lower the risk of sustaining musculotendinous injuries.

Limitations: The result of this study should be interpreted with caution as the relatively small sample size is going to influence generalization. Further study with proper follow up could be conducted to find out the long-term effects of CRAC stretching.

CONCLUSION

The findings of our study have suggested that enhanced flexibility could be observed with an accompanying decrease in the number of hamstring curls performance in response to self-administered CRAC stretching. These findings are unique in that, to our knowledge, no other authors have examined the effects of CRAC stretching on isolated muscle performance with a repeated measures design. Regarding the mechanisms underlying the stretching-induced performance deficit, the decreases in no of hamstring curls we observed in our study tentatively support the hypothesis that stretching may alter the length-tension relationship and the hypothesis that stretching may reduce muscle activation, respectively. CRAC stretching can be made part of a full warm-up routine because of its positive impact on flexibility and musculotendinous injury occurrence in the physically active older population.

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Preparation of Silver Nanoparticles (AgNPs) using Different Plants and Their Antibacterial and Antifungal Properties: A Review

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ABSTRACT

Since last decades, silver or its salt related materials applied as antimicrobial agents, water purification in clinics, in burn dressing. In addition, unique characters of AgNPs have leads to several study such as applications in engineering, nanomedicine, bio-based materials, clean energy, and food industry. Silver based compounds are more cost effective than other metal nanoparticles. Silver nanoparticles (AgNPs) has been documented as a widely used as antimicrobial agent for decades. AgNPs shown outstanding capacity in a various type of biological applications including antibacterial, antifungal, anti-parasites, anticancer etc. There are several methods for the synthesis of AgNPs such as physical, chemical and biological method. In physical and chemical methods production of unwanted hazardous by-product, use of high energy and problem in stability. Biological mode of synthesis is popular and advantageous over other methods. Therefore, there is urgent requirement for greener methods of preparation of AgNPs are gaining attention day by day. The main objective of greener synthesis of AgNPs is to decrease toxic by-products. In addition, these greener methods are cost-effective and in the abundance of reducing raw agents. In order to control the shape and size of AgNPs synthesis routes should be carried out in controlled ambient temperature and under near physiological pH. One of the major application of AgNPs is antimicrobial properties including antibacterial activity against multi-drug resistant bacteria and antifungal. This review dedicates a brief outline of the research on green synthesis of AgNPs and the impact of the protocols on their shape, size and morphology and its antimicrobial properties.

KEY WORDS: SILVER NANOPARTICLES, GREEN SYNTHESIS, ANTIBACTERIAL, ANTIFUNGAL PROPERTIES.

INTRODUCTION

In the scientific community, nanotechnology field shown very significant role in modern research area which are dealing with engineering and manipulation of structure of particles at nanoscale which size ranges from 1 to 100 nm (Sanchez et al., 2010). At nano scale the properties of nanostructures such as physiochemical and biological transformations in unique ways when compared with their corresponding bulk (Baer et al., 2010). Preparation of nanoparticles can be carried out by either physical, chemical or biological methods (Rycenga et al., 2011). But synthesis of nanoparticles using biological inspired methods are more easy and fruitful as compared to others (Raghunandan et al., 2010, Bahadur et al., 2011).

Applications of nanomaterial's are depending on various size, shape, uniformity, composition and topography (Chen et al., 2013). Using of nanoparticles in diverse areas including electronics (Rubilar et al., 2013), fashion

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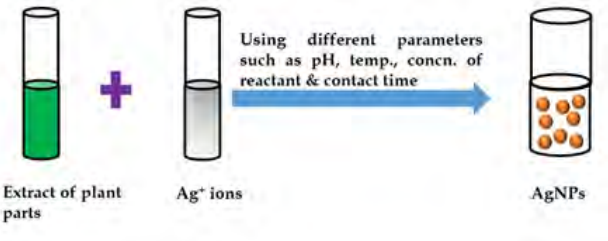
industry (Sonkaria et al., 2012), biomedical (Mishra et al., 2013, Ravindran et al., 2013), drug or gene delivery (Sripriya et al., 2013), sustainable environment (Hebbalalu et al., 2013), biomechanics (Subbiah et al., 2013), optics and lenses (Austin et al., 2014), biochemical and chemical manufacturing (Borase et al., 2014, Rajkumar et al., 2016), catalysis (Kästner et al., 2016), florescence (Anfossi et al., 2018), and photo-electrochemical (Guitoume et al., 2018, Sharma et al., 2019).

The nanoparticles can be applied in diverse areas for different purposes, but the AgNPs believed as the most encouraging nanostructures due to advent of significant antibacterial properties (Kotcherlakota et al., 2019, Ravi et al., 2019). AgNPs are more potentially antimicrobial because of their large surface area to volume ratio when compared to bulk silver metal (Fahimirad et al., 2019). AgNPs are also gain interest in scientific community due to the growing concern regarding increase in antibiotics resistance against several microbial strains (Menazea et al., 2020, Salleh et al., 2020). For nanomedicine applications such as in wound dressings, topical creams for burning case etc. AgNPs showed biocidal effect against several types of microorganisms (Garibo et al., 2020).

Biosynthesis of AgNPs: Biosynthesis of AgNPs using plants: For the first time AgNPs were synthesized using alfalfa sprouts i.e. using a living plant system (Marchiol et al., 2014). When biosynthesis of AgNPs using plant is compared to microorganisms including bacteria and fungi, plants looks better option. Plant/plant parts extracts are able to generate Ag NPs very rapidly (Chokriwal et al., 2015). By the virtue of simplicity, easy to handle and readily availability, biosynthesis of AgNPs was maximum achieved using plants (Khatami et al., 2016). Judicious selection of the plant extract, and the major exceptional affecting surrounding parameters are the concentration of the plant extract and metal salt, the temperature, the pH, and the incubation time (Anjum et al.,2016). By controlling the synthesis parameters, desired shape and size of AgNPs can be achieved. Plant parts such as fresh or dried leaves, roots, latex, gum, bark, stem, and seeds are being used for nanoparticle synthesis. Schematic representation of synthesis of AgNPs using plant extract is demonstrated by Figure 1.

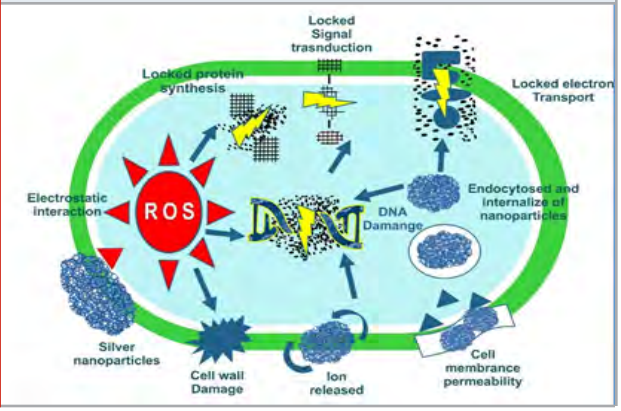
Figure 1: Illustration of biosynthesis of AgNPs using plant extract



Plant extract contains active biomolecules leads to reduction and stabilization process during synthesis process of AgNPs (Rajeshkumar et al., 2017). Active biomolecules including phenolics, polysaccharides, flavonoids, alkaloids, proteins, enzymes, and amino acids. The phytochemicals such as flavonoids and phenols have exceptional capability to reduce AgNPs, which inhibits agglomeration (Selvan et al., 2018). Mishra et al., (2013), shown biosynthesis of AgNPs using neem leaf extract and TEM observation showed size of nanoparticles ranges from 2-8 nm. Further, AgNPs was characterized by several standard techniques such as Surface Plasmon Resonance Spectra, Fourier Transform Infrared Spectroscopy (FTIR), Circular Dichroism (CD), Dynamic Light Spectroscopy (DLS), and Surface Tunneling Microscopy (STM). Ashoka leaf extract was also used for synthesis of AgNPs. This AgNPs was used as antiplasmodial agent (Mohammadi et al., 2019). Recently, enhanced antibacterial properties shown by ultrasonic-assisted green synthesis of AgNPs using *Mentha aquatica* leaf extract (Nouri et al., 2020). Another paper shown by Uddin et al., (2020) showed *Cocos nucifera* leaf extract mediated preparation of AgNPs (14.2 nm) for enhanced antibacterial activity. Table I. shows plant extract mediated synthesis of AgNPs.

Antimicrobial activity of plant mediated AgNPs: AgNPs has been extensively used across the scientific world for several applications. Ag+ is a famous antimicrobial agent which has been used against approx. 650 microorganisms including bacteria, fungi or viruses (Boateng et al., 2020). It was observed that AgNPs has the outstanding antibacterial activity and is minimum toxic to animal or human cells. Anticipated mechanism of AgNPs against bacteria is described in Figure 2.

Figure 2: Illustration of antibacterial activity of A¬¬gNPs (Vega-Jiménez et al., 2019)



Antibacterial activity of AgNPs: Few research papers describing the electrostatic attraction between +ve charged AgNPs and -ve charged bacterial cells. AgNPs may gathered inside the plasma membrane of bacterial cells leads to damage cell wall or membranes (Erol et al., 2020). It is speculated that Ag atoms may bind to thiol groups (-SH) of protein/enzymes and thus forming stable bonds. Binding of silver atoms with –SH group causes the deactivation of proteins/enzymes in the cytosol or biological.

Table 1. Use of Silver (Ag) NPs as an Antibacterial agent

S.N.	Method of Synthesis	Size of Silver (Ag) Nanoparticle (nm)	Name of Bacteria	Ref.
1	*Gum kondagogu*	18.9-55.0	*Staphylococcus aureus*	Kora et al., 2010
			E. coli	
			Pseudomonas aeruginosa	
2	*Acalypha indica* Leaf	20-30	*E. coli*	Krishnaraj et al., 2010
			Vibrio cholerae	
3	*Carob Leaf* Extract	5-40	*E. coli*	Awwad et al., 2013
4	Mango Peel Extract	7-27	*E. coli*	Yang et al. 2013
			S. aureus	
			B. subtilis	
5	*Artocarpus heterophyllus* Lam.	10.7	*Bacillus cereus*	Jagtap et al.,2013
			Bacillus subtilis	
			Pseudomonas aeruginosa	
6	*Coffea arabica* seed extract	20-30	*E. coli*	Dhand et al., 2016
			S. aureus	
7	Medicinal plant leaf extract	10-18	*E. coli*	Jain et al., 2017
8	*Thymus kotschyanus* extract	50-60	*E. coli*	Hamelian et al., 2018
			P. aeruginosa	
			S. aureus	
			B. subtilis	
9	*Berberis vulgaris*	30-70	*E. coli*, *S. aureus*	Behravan et al., 2019
10	*Lysiloma acapulcensis*	5	*E. coli*	Garibo et al., 2020
			S. aureus	
			P. aeruginosa	

Table 2. Use of Silver (Ag) NPs as an Antifungal agent

S.N.	Method of Synthesis	Size of Silver (Ag) Nanoparticle (nm)	Name of Fungi	Ref.
1	*Croton sparsiflorus* morong	22-52	*Mucor Sp*	Kathiravan et al., 2015
			Tricoderma sp	
			Aspergillus nigar	
2	*Mentha pulegium*	-	*Candida albicans*	Abd Kelkawi et al., 2016
3	*Pelargonium/Geranium leaf extract*	29	*Aspergillus flavus*	Mohammadlou et al., 2017
			Aspergillus terreus	
4	Grass waste	15	*F. solani*	Khatami et al., 2018
			R. solani	
5	Starch	-	ERG11	Prasher et al., 2018
6	*Rosa canina*	13-21	*Candida albicans*	Gulbagca et al., 2019
7	*Citrus limetta* peel extract	18	*Candida species*	Dutta et al., 2020
8	*Ferulago macrocarpa* flowers extract	14-25	*Candida albicans*	Azarbani et al., 2020
9	*Teucrium polium* L.	10 -100	*F. oxysporum.*	Ghojavand et al., 2020
10	*Phyllanthus urinaria*, *Pouzolzia zeylanica*, and *Scoparia dulcis* Leaf Extracts	26.7	*Aspergillus niger*	Nguyen et al., 2020
			Aspergillus flavus	
			Fusarium oxysporum	

Membrane which cause inhibition of physiological processes such as trans membrane ATP production and ion transport (Makvandi et al., 2020) Table I. shows different plant mediated synthesis of AgNPs and its antibacterial application in different strains of bacteria.

Antifungal activity of AgNPs: Due to the regular increase of drug resistance in clinical strains of fungi, the researchers' communities and pharmaceutical companies are exploring for novel antifungal agents such as AgNPs (Varier et al., 2019) Plant extract derived AgNPs showed higher antifungal activities clinical fungal pathogens when compared with the presently available antifungal drugs. Antifungal properties of AgNPs was tabulated in Table II.

CONCLUSION

Cleaner production of AgNPs using green chemistry route is demanding to improve and maintain sustainable environment. Advantages of fabrication of AgNPs including cost effective, easy to synthesis at large scale, taking lesser time as compared to other processes. Synthesis of AgNPs using plants could be beneficial over other biological based materials. As a matter of fact, identification and characterization of biomolecules from plant extract which are solely responsible for synthesis and stabilization of AgNPs will be the crucial to overcome the problems. Biosynthesized AgNPs shows excellent antibacterial and antifungal activity. The biocidal activity of AgNPs against pathogenic bacteria and fungi with minimum toxic effects on normal cells could open a new avenue of research in the nanomedicine area.

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