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The central roles of exosomes in hematological malignancies: A new frontier review

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ABSTRACT

Exosomes, which are 30- to 120-nm vesicles, are released by most types of cells, including tumor cells. In hematological malignancies, exosome-mediated expulsion of a number of key proteins and micro RNAs, resulting in influence of major tumor-related pathways. Emerging evidence suggests that the component secreted by exosomescan promote tumor survival,angiogenesis and metastasis, and also mediate tumor microenvironment induced drug resistance and immune escape. Furthermore, exosomes contain a great variety of bioactive molecules and are emerging as rich reservoirs of hematological tumor-specific biomarkers for the detection and therapeutics.This comprehensive review highlights the advancements in understanding of the pathogenesis of exosomes secretion and the consequence onhematological malignancies development. Full knowledge of the contribution of exosomes to the potential medical application ofdiagnosis and treatment will depend on the ingenuity of future investigators and their insight into biological processes.

KEY WORDS: HEMATOLOGICAL MALIGNANCIES; EXOSOMES; DISEASE DEVELOPMENT;DIAGNOSIS; TREATMENT

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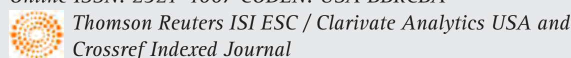

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INTRODUCTION

Hematological malignancies, which include lymphoid, myeloid, histiocytic, and mast cell neoplasms, are a heterogeneous group of diseases of diverse incidence, pathogenesis and prognosis (Kornblau *et al.*, 1998). The prognosis of hematological malignancies patients diverges greatly, largely depending on thepathological types of the patient (Li *et al.*, 2016). Nonetheless, enhancing understanding of tumorigenesis mechanism is critical for development of novel diagnosis and therapeutic strategy. Exosomes are a kind of extracellular vesicles (EVs), which was first reported by Pan and Johnstone in 1983 as unwanted cellular components extruding from reticulocytes. EVs are medium-sized vesicles, ranging from 30 to 120 nm and are secreted by different cell types under both physiological and pathological conditions. Exosomes are rich in cholesterol, sphingomyelin, ceramide lipids, protein, mRNA, miRNA, and various other signaling molecules from donor cell. In addition, CD9, CD63, and CD81 are most frequently detected and are considered asthe classic markers of exosomes, (Malla *et al.*, 2018).

Then, the MVB's can either fuse with lysosomes resulting in degradation of intra-luminal contents or they can secrete their content as exosomes outside the donor cells. Furthermore, there are several ways by which exosomes are taken up by recipient cells: receptor- or lipiddraft-mediated endocytosis, phagocytosis, macropinocytosis, or fusion with the plasma membrane of a target cell, For transportation, exosomes mainly originate from multi-vesicular bodies (MVB's) in the cells which are produced by the invagination of endosomal limiting membrane (Pfrieger *et al.*, 2018).

Recently, it has been reported by many workers that the potential functions of exosomes contributed to various aspects of hematological tumorigenesis, particularly with a focus on the exosome-mediated tumor progression, metastasis, drug resistance and immune escape by altering the function of receiver cells via diverse exosomal cargoes including proteins, DNA, messenger RNAs (mRNAs), and microRNAs) (Whiteside and Boyiadzis, (2017). Furthermore, exosomes are found in most biological fluids including urine, blood, ascites, saliva and cerebrospinal fluid, which can be made for very attractive targets for diagnostic application.Although the known information is limited, exosomes have been reported to play an emerging role in various aspects of the tumor survival, metastasis, drug resistance, and immune escape of hematologic tumor. In this review, we will focus specifically on the effects of exosomes on tumorigenesis, diagnosis and treatment of hematological malignanciesand the relevantnew prospects.

THE ROLE OF EXOSOMES IN DEVELOPMENT OF HEMATOLOGICAL MALIGNANCIES

Recently, considerable amount of studies have revealed that exosomes were secreted by tumor cells ortumor micro-environment cells and cross-talk influence tumor proliferation, angiogenesis, drug resistance and immune escape of several hematological cell types (figure1) (Sun, *et al.*, 2018).

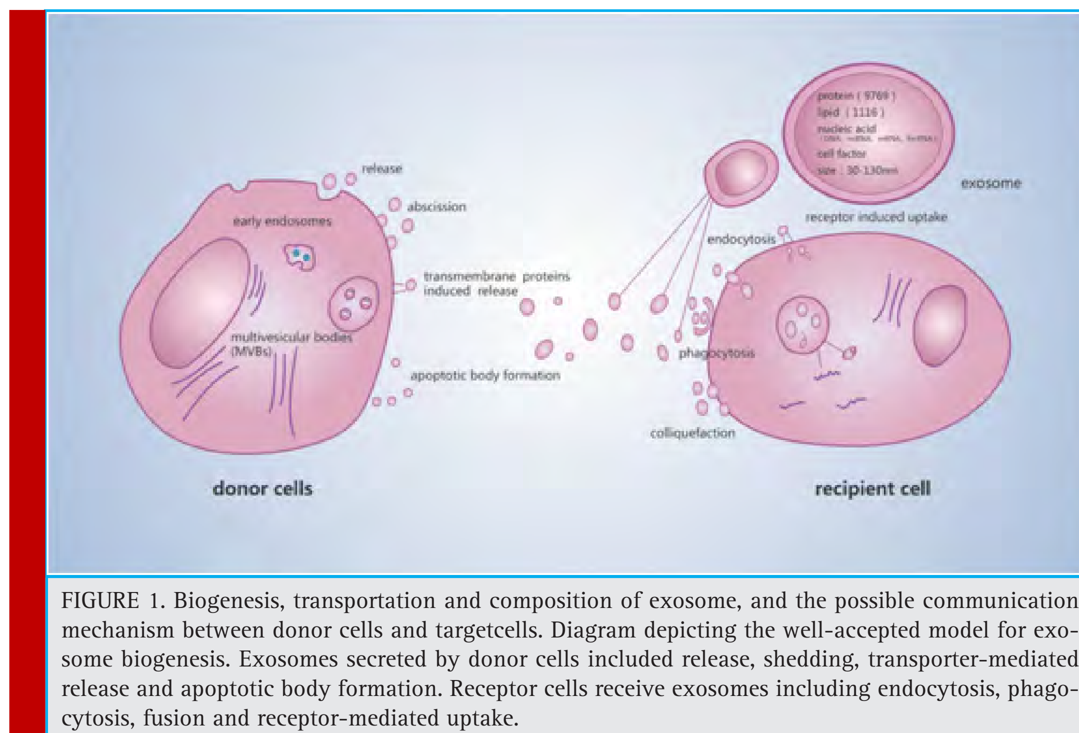

FIGURE 1. Biogenesis, transportation and composition of exosome, and the possible communication mechanism between donor cells and targetcells. Diagram depicting the well-accepted model for exosome biogenesis. Exosomes secreted by donor cells included release, shedding, transporter-mediated release and apoptotic body formation. Receptor cells receive exosomes including endocytosis, phagocytosis, fusion and receptor-mediated uptake.

Survival and proliferation

One study demonstrated that the level of sera-derived exosomes in acute myeloid leukemia (AML) patients are higher than sera-derived exosomes from healthy controls [Hong *et al.*, 2014] and the exosomes secreted by AML cells express higher level of mRNA, such as GATA1, FOX3, SHIP1, ID1, E2F1, CEBP-α, CEBP-β, Myc and MEF2C, which are the group of transcripts of genes to the development of AML [Huan J. *et al.*, 2013]. Furthermore, another study showed that levels of active TGF-β1 carried by exosomes obtained at AML diagnosis were high and then decreased following induction chemotherapy. In addition, in long-term remission of AML patients, TGF-β1 levels in plasma-derived exosomes approach the same levels seen in exosomes of normal controls. The results indicate that exosomal protein and TGF-β1 levels in AML plasma could serve as biomarkers of response to chemotherapy.These data showed that both AML cell lines and primary AML blasts released exosomes relevant to AML pathogenesis (Huan *et al.*, 2013 and Long *et al.*, 2017).

For chronic myelocytic leukemia (CML), CML cell line LAMA84-derived exosomes increase levels of IL-8 mRNA and protein in HS-5 (bone marrow stromal cell line), and further promote HS-5 induced CML cell proliferation, (Zhou *et al.*, 2012). In another way, the exosomes also reduce expression of the proapoptotic genes BAD, BAX and PUMA and increase expression of anti-apoptotic genes BCL-xL, BCL-w, and BIRC5.Moreover, exosomes also activate the PI3/AKT and MAPK/ERK signaling pathways. Collectively, the exosomes secreted by CML cells both educate the tumor microenvironment cells and directly affected the proliferation and apoptosis of LAMA84 cells and finally promote the CML cells survival. Human T-lymphotropic virus type 1 (HTLV1) infected T cells release exosomes that contain viral Tax protein and Tax, HBV, and EBV mRNA, which increase levels of phosphorylated AKT and active NF-kB pathway, and further facilitate T-cell tumor cells survival. These results suggest that the exosomes released from HTLV-1-infected cells play key role in the pathogenesis of T-cell leukemia (Jaworski *et al.*, 2014, Raimondo *et al.*, 2015).

The study by Raphael Koch shows that diffuse large B-cell lymphoma (DLBCL) possess a self-organized infrastructure comprising side population (SP) and non-SP cells, where transitions between clonogenic states aremodulated by exosomes mediated Wnt signaling. Lymphoma SP cells exhibit autonomous clonogenicity and export Wnt3a via exosomes to neighboring cells, thus modulating population equilibrium in the tumor (Koch *et al.*, 2014). The study about the role in MCL observes that MCL exosomes are taken up rapidly and preferentially by MCL cells. Only a minor fraction of exosomes was internalized into T-cell leukemia and bone marrow stroma cell lines, when these cells werecocultured with MCL cells. Moreover, MCL patients' exosomes were taken up by both healthy and patients' B-lymphocytes with no apparent internalization to T lymphocytes and NK cells. Exosome internalization was not inhibited by specific siRNA against caveolin1 and clathrin but was found to be mediated by a cholesterol-dependent pathway. These findings demonstrate natural specificity of exosomes to B-lymphocytes and ultimately might be used for therapeutic intervention in B cells malignancies, (Hazan *et al.*, 2015).

EBV infection of B cells in vitro induces the release of exosomes that harbor the viral latent membrane protein 1 (LMP1). LMP1 via exosomes actives CD40 signaling and induces proliferation of B lymphocytes and T cell independent class-switch recombination.Finally, LMP1 drove B cell differentiation toward a plasmablast-like phenotype. In conclusion, the results suggest that exosomes released by EBV-infected lymphoma cells include the production of the activation-induced cytidine deaminase (AID), and further promote tumor cells aggressive and progression, (Nanbo *et al.*, 2013). In addition, Chugh *et al.* (2013) reported that exosomes derived from patients with KSHV-associated malignancies and KSHV mouse modelscontained KSHV encoded microRNAs such as miR-17-92 cluster, which are affect the targets of KSHV signaling pathways that may therefore be part of the paracrine signaling mechanism that mediates KSHV pathogenesis.

A research about relationship between MSC and plasma cells demonstrated that the tumor-supportiverole of BMSC-derived exosomes: Compared with normal mesenchymal stem cells, BMSC-derived exosomes in MMexpresslower level of microRNA15a and microRNA15a isa tumor-suppress factor thatcontributeto MM disease progression. In addition, BMSC-derived exosomes in MM expresshigher content levels fibronectin, indicating that BM-MSC-derived exosomes may differentially impact MM cell adhesion, (Roccaro *et al.*, 2013). Kim De Veirman *et al.* (2016) showed that miRNA-146a in MM-derived exosomes can be transmitted into mesenchymal stem cells, resulting in induction of expression for some cytokines and chemokines including CXCL1, IL6, IL-8, IP10, MCP-1, and CCL-5, thus lead to increasing vitality and progression of MM cells.

Angiogenesis and metastasis:Exosomes have been increasingly recognized as a new mediator for angiogenesis and metastasis of hematological malignancies. For example, Umezu *et al.*(2013) showed that leukemia cells K562 released the exosomal miRNAs, such as miR-17-92 cluster, especially miR-92a, into human umbilical vein endothelial cells (HUVECs) ,and target reduced the expression of integrin α5 in HUVECs by exosomal

miR-92a, indicating that exogenous miRNA via exosomal transport can function like endogenous miRNA in HUVECs, which enhance endothelial cell migration and tube formation. In the study, which investigated the angiogenic role of exosomes produced by acute promyelocytic leukemia cells NB4.PML-RARA transcript has been detected in NB4 exosomes and taken up by endothelial cells, resulting in decreasing the levels of VEGF and tissue factor (TF) through increasing IL-8 mRNA and protein content in their EVs, renders the HUVECs more TF-positive and procoagulant, (Fang *et al.*, 2016).

A recent study by Hiroko Tadokoro *et al.* has clearly demonstrated that hypoxia promotes the release of exosomes in K562 cells,the amount of exosomal miR-210, which down-regulated EFNA3, an inhibitor of angiogenesis, (Tadokoro *et al.*, 2013). The results suggest that exosomal miRNA derived from cancer cells under hypoxic conditions may partly affect angiogenic activity in endothelial cells. Paggetti *et al.* found that CLL-derived exosomes are actively incorporated by endothelial and mesenchymal stem cells ex vivo and in vivo and that the transfer of exosomal protein and microRNA induces an inflammatory phenotype in the target cells, which resembles the phenotype of cancer-associated fibroblasts (CAFs), (Paggetti *et al.*, 2015).

Exosomeswere uptakenby endothelial cells increased angiogenesis ex vivo and in vivo, and coinjection of CLL-derived exosomes and CLL cells promoted tumor growth in immunodeficient mice. These findings demonstrate that CLL-derived exosomes actively promote disease progression by modulating several functions of surrounding stromal cells that acquire features of cancer-associated fibroblasts, (Paggetti *et al.*, 2015). Another similar study found that EBV-positive Burkitt's lymphoma cells Raji released exosomes with miR-155 inducing angiogenesis in remote recipient cells, whereas no major difference was found in co-culture with EBV-negative Burkitt's lymphoma cells, (Yoon *et al.*, 2016).

Thus, it would be reasonable to believe that specific viral exosomal microRNAs contribute to angiogenesis of vascular endothelial cells, subsequently leading to pathophysiologic angiogenesis. In accord with a study by Umezu *et al.* (2014), miRNA-135b from MM-derived exosomes accelerated HIF-1 transcriptional activity via inhibition of FIH-1, which is called the HIF-FIH signaling pathway, exertinganangiogenesis influence. Collectively, these results suggest that hematological malignancy cells release exosomes that could promote tumor metastasis and the formation of pre-metastatic niches to create a microenvironment favorable to survival and proliferation of tumor cells themselves.

Drug resistance: Like many other tumors, increasing evidences also revealed that the tumor microenvironment (TME) has crucial impact on hematological malignancies initiation and progression (Rizzo *et al.*, 2011). Given the central role of exosomes in cellular communication, it is undoubtable that exosomes also contribute to microenvironment-induced drug resistance. One intuitive mechanism involving exosomes would be the sequestration of cytotoxic drugs in the intracellular vesicles and subsequent expulsion, to negate drug effect within the cells. Notably, emerging evidence suggests that both the exosomes released from TME cells and the tumor cells themselves help the hematological malignancy cells to resist chemotherapy, (Isidori *et al.*, 2014). One prominent example, exosomes from both AML-BMSC and healthy controls protect MOLM-14 FLT3 internal tandem duplication (FLT3-ITD+) AML cells from cytarabine and stromal exosomes alter chemo-resistance in AML cells. Further, only AML-BMSC exosomes significantly protected AML cells from the FLT3 inhibitor AC220 after exposure. The protection might be associated with elevated level of miRNA-155, miRNA-375, cytokine epidermal growth factor (EGF) and TGF-β1 (Viola *et al.*, 2016).

Finally, these data imply a few novel approaches to overcome drug resistance on AML blasts by either blocking exosome-leukemia cell communication, or inhibiting tumor microenvironment exosome production. Moreover, several studies unveiled that galectin-3 was upregulated and stabilized anti-apoptotic Bcl-2 family proteins in survival leukemia cells, which facilitates escape from apoptotic stimuli through activation of Wnt/β-Catenin signaling pathway and the PI3K/Akt pathway, (Cheng *et al.*, 2011, Hu K. *et al.*, 2015).

In another study about lymphoma, the results revealed that high levels of adenosine triphosphate (ATP)-binding cassette (ABC) transporter A3 (ABCA3) were related to drug resistance, especially by drug expulsion which might be modulated by microparticles (Chapuy *et al.*, 2008, Steinbach, *et al.*, 2006). Oksvold *et al.* (2014) showed a similar observation that exosomes secreted by B-cell lymphoma reduced rituximab-induced cytotoxicity. These studies indicated a novel mechanism of drug resistance in lymphoma, which is linked to an ABCA3-dependent pathway of exosome secretion, (Oksvold *et al.*, 2014). In particular, it was suggested that increased expression of cellular galectin-3 and elevated concentration of galectin-3 in circulating system may contribute to tumor progression and drug resistance. Regarding ALL, the levels of exosomal Gal-3 mRNA and protein, which originated from stromal cells in B-ALL were in relation with the appearance of drug-resistance through activated MEK/ERK pathway, (Fortuna *et al.*, 2014). Galectin-3 is enriched in OP9 exosomes, and exosomal galectin-3 can be internalized by ALL cells, and activates NFκB signaling pathway, whichisoften linked to anti-apoptosis and drug resistance (Hu *et al.*, 2015).

Another study showed that exosomal evasion of humoral immunotherapy in aggressive B-cell lymphoma wasmodulated by ATP-binding cassette transporter A3. B-cell lymphoma cells released exosomes that carried CD20, bound therapeutic anti-CD20 antibodies, consumed complement, thereby impairing antibody-dependent cell mediated cytotoxicity (ADCC) and protected target cells from antibody attack through linked in an ABCA3-dependent pathway of exosome secretion. Additionally, removing exosomes from plasma samples of B-NHL resulted in considerable improvements in the effect of rituximab against lymphoma cell in vitro, (Aung *et al*., 2011). In multiple myeloma, the integrin-mediated interaction of cancer cells with MSC, upregulates the secretion of soluble factors such as IGF-1, FGF, IL-6 and others that provide growth advantage and drug resistance to multiple myeloma cells (Isidori *et al*., 2014, Greco *et al*., 2009).

Another study by Wang *et al.* (2014) indicated that bone marrow stromal cell–derived exosomes in MM can promote proliferation, migration, and survival of MM cells, andcan also induce chemotherapy drug resistance to bortezomib, influencing several pathways including c-Jun N-terminal kinase, p38, p53, and Akt which are relevant to survival of MM cells.All these studies suggest that exosome mediate drug resistance through both the direct shuttling of drugs out of the cells and the horizontal transfer of molecules/molecular signals that bestow drug resistance to the otherwise sensitive cells.

Immune escape: Hematological malignancy cells can evade host immune surveillance, a well-known phenomenon called as immune evasion or immune escape, which is also a hallmark of tumor. Many research groups have demonstrated abundant quantity of exosomes released by tumor cells exerting an immunosuppressive effect that helps them evade immune response. Dysfunctions of natural killer (NK) cells, which are a major component of the anti-tumor immune response, have been reported in various hematologic malignancies, including chronic lymphocytic leukemia (CLL). BAG6, which issecreted by tumor cell, activate receptor NKp30 on the surface of NK cells, further increase NK cell cytotoxicity and promote NK cells to kill cancer cells , and is suggested that down-regulated or absent of exosomal BAG6 in CLL patients evasion of CLL cells from NK cell anti-tumor activity, (Reiners *et al*., 2013).

TGF-β1 is a potent immunosuppressive molecule that inhibits NK cell cytotoxicity. The serum concentration of exsomal TGF-β1 in the newly diagnosed AMLwas-significantly higher than that of the normal control. Furthermore, NK cell differentiation is IL-15 dependent, and IL-15 plays a major role in NK-cell expansion and promotes NK-cell survival, IL-15 is also able to counteract immunosuppressive effects mediated by TGF-β carried on exosomes from AML patients. Taken together, these evidence suggest IL-15 can enhance anti-tumor effects of NK cells in AML patients, (Greco *et al*., 2009, Hong *et al*., 2014). In virus-related lymphomas, EBV+ lymphoma cells are embedded in non-neoplastic by standers: B and T cells, macrophages. There is increasing evidence that the indirect actions (i.e. immunosuppression and TME components) of different viruses also play significant roles in lymphomagenesis, (Esau, 2017).

For example, EBV and HIV-1 are proved capable of inducing the overexpression of PD-L1 on antigen-presenting cells, thus resulting in immunosuppression by the increased apoptosis of T cells. Furthermore, the EBV-miRNAs in the exosome secreted from EBV positive lymphoma cells were transferred to macrophage and promote the development of lymphoproliferative disease (LPD) in vivo mouse model, (Lichterfeld *et al*., 2008). Another study showed that the exosomes present in the serum of CHB patients contain both HBV nucleic acids and HBV proteins, HBV might influence NK-cell function via exosomes through upregulating immunosuppressive factors, such as TGF-β, in HBV-infected CHB patients. These observations suggest that exosomes may serve as important regulators of HBV transmission and may be involved in escaping innate immunity.

THE ROLE OF EXOSOMES IN DIAGNOSIS AND PROGNOSIS OF HEMATOLOGICAL MALIGNANCIES

Early cancer detection and disease stratification or classification are critical to successful treatment. Accessible, reliable, and informative hematological malignancies biomarkers can be medically valuable and can provide some relevant insights into cancer biology [Elsherbini and Bieberich., 2018]. Early detection of cancer could be easily performed using exosomes isolated from body fluids such as blood plasma, serum, and urine, which is allowing for a non-invasive method of detection of hematological malignancies, (Kang *et al*., 2018).

Microvesicles are found in higher concentrations in the sera of different types of hematological malignancies, such as AML, ALL, CML, CLL, diffuse large B-cell lymphoma (DLBCL), Hodgkin's lymphoma (HL) and MM patients and abundantly express surface proteins unique to their cell of origin, which is rarely observed from serum microvesicles of normal controls [Kang *et al*., 2018]. AML biomarkers NPM1, FLT3, CXCR4, MMP9 and IGF-IR are also present in AML cell derived exosomes, along with mRNAs involved in leukemia development(Boyiadzis, 2016). Hong. *et al* (2014). indicated that exosomal TGF-β1 levels and relative levels of the three TGF-β1 forms (TGF-β1 pro-peptide, latency-associated peptide (LAP), and mature TGF-β1 were distinct in AML patients in

Table 1. The role of exosome in the development of hematologic tumor.				
Targets	Type	Disease	Function	Reference
TGF-β1 MICA, MICB,ULBP1,ULBP2, BAG6	protein	AML	Reduce the ability of natural killer (NK) cells to kill leukemic cells.	Cheng *et al.*, 2011 Jaworski *et al.*, 2014
GATA1, FOX3, SHIP1, ID1, E2F1, CEBP-α, CEBP-β, Myc, MEF2C	protein	AML	Induce development of leukemia.	Malla *et al.*, 2018.
PI3/AKT,MAPK/ERK, NF-kB,TGF-β1	protein	CML	Enhance cell survival of LAMA84 cell	Sun *et al.*, 2017
Tax, AKT, Rb, cFLIP, NF-kB		ATL	Enhance cell survival in murine and human T-cell cell lines.	Hong *et al.*, 2014
Galectin-3, NF-kB	protein	ALL	Promote ALL drug resistance.	Hong *et al.*, 2014
SRC, TSP-1, IL-8	protein	CML	Promotes endothelial cell angiogenesis.	Nanbo *et al.*, 2013
MiR-210	microRNA	CML	Promote the vascular activity of CML.	Nanbo *et al.*, 2013
Wnt3a	protein	DLBCL	Promote the growth of DLBCL cells.	Huan *et al.*, 2014
IL-6, CCL2, fibronectin	protein	MM	Promote the growth of MM cells.	Raimondo *et al.*, 2015
miR-135b	microRNA	MM	Promoteendothelial vessel formation.	De *et al.*, 2016
miR-34a, miR-125b- 5p, miR-146a, miR-15a, miR -137/197, miR- 21	microRNA	MM	Facilitate multiple myeloma growth and progression	Nanbo *et al.*, 2013 Jaworski *et al.*, 2014

AML: acute myeloid leukemia; CML: chronic myelocytic leukemia; ATL: T-cell acute lymphoblastic leukemia; ALL: acute lymphoblastic leukemia; DLBCL: diffuse large B-cell lymphoma; MM: multiple myeloma

different stages of chemotherapy[Hong C.S. *et al.*, 2014]. This stability makes exosomes as suitable mines for hunting reproducible and consistent biomarkers. ALL-associated expression of miRNA92a can be detected in the circulating vesicles of a majority of ALL patients (Rekker K. *et al.*, 2014)

In CML, Mineo *et al.* (2012), showed that a co-evolution between endothelial cells and CML cells are essential

Table 2. Circulating vesicles as a general hematological malignancies biomarker.				
Biomarker	Type	Source	Hematological Malignancies	Reference
NPM1, FLT3, CXCR4, MMP9 and IGF-IR	protein	serum	AML	Wang *et al.*, 2014
MiR-92a	MicroRNA	serum	ALL	Hong *et al.*, 2014
TGF-β1	protein	serum	AML	Reiners *et al.*, 2014
miR155	MicroRNA	serum	AML, CLL, WM, MDS, MM	Elsherbini & Bieberich 2018
Src	protein	serum	CML	Hong *et al.*, 2014
MiR-155, MiR-210, MiR-21	MicroRNA	serum	DLBCL	Rekker *et al.*, 2014
MiR-22	MicroRNA	plasma	HL	Caviano *et al.*, 2014
MiR-15a, let-7b miR-18a,	MicroRNA	plasma	MM	Caviano *et al.*, 2014

AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia; WM: macroglobulinemia of Waldenstrom; MDS: myelodysplastic syndrome; MM: multiple myeloma; CML: chronic myelocytic leukemia; DLBCL: diffuse large B-cell lymphoma; HL: Hodgkin's lymphoma.

for leukemia progression and resistance to therapy. This is possible because of the fact that K562 malignant cells secret growth factors and various miRNAs and transport these 'endothelial inducing factors' via exosomes. As a result, tube formation is stimulated, even when treating with imatinib, a tyrosine kinase inhibitor that targets the Philadelphia chromosome-positive (Ph+) myeloid leukemia cells. In this case, the development of angiogenesis was reported to regulate the progression and dissemination of this hematological malignancy, (Wojtuszkiewicz *et al*., 2016 Caivano *et al*., 2017).

Parikh *et al.* (2016) found the EV miR155 level may serve as a promising prognostic/predictive biomarker in CLL, independent of clinical stage. In conjunction with the previously reported data, Caivano *et al.* (2017) found that the EV miR155 levels were significantly higher in CLL, AML and Waldenström'smacroglobulinemia (WM) cases compared to controls (Parikh *et al*., 2016, Zhu *et al*., 2018). Conversely, they also found that the EV miR155 levels were significantly lower in myelodysplastic syndrome (MDS) and MM cases, (Caivano *et al*., 2017). In addition, they found that high EV miR155 levels correlated with high white blood cell counts in AML patients. In conclusion, this study indicated that EV miR155 may serve as an attractive new, non-invasive diagnostic biomarker in human hematologic malignancies. Levels of exosomal miR-155, miR-210 and miR-21 in serum from DLBCL patients (n=60) were higher than control sera (n=43)(P=0.009), (Mineo *et al.* 2012).

Monique *et al.* found that EV-associated miR21-5p, miR127-3p, let7a-5p, miR24-3p, and miR155-5p signals were higher in primary and relapsed classic Hodgkin's lymphoma (cHL) patients compared with healthy individuals [van Eijndhoven M.A. *et al*., 2016]. However, they also detected that miR21-5p and miR155-5p is small, but signifcant, decreases (2-fold, P = 0.016) in plasma after therapy. Nevertheless, the decrease in miR155-5p was more pronounced in the EV fraction, (4-fold, P = 0.016). They detected high levels of miR127-3p in EVs produced by HRS cells in plasma EVs of cHL patients but less abundant levels in healthy control EVs, which suggests that the pool of miR127-3p detected in the protein fraction is unrelated to cHL tumor tissue and is derived from other sources [van Eijndhoven M.A., *et al*., 2016]. miR-15a is lower in mesenchymal stromal cells derived vesicles of MM patients compared with healthy subject, and miRNA-rich exosomes secreted from MM-mesenchymal stromal cells facilitate MM progression, (Manier *et al*., 2017).

Lower expression of let-7b or miR-18a was significantly associated with a high ISS stage. However, both let-7b and miR-18a were independent predictors after adjusting for the ISS and specific cytogenetic abnormalities. The effect of the two miRNAs on PFS and OS was illustrated by Kaplan-Meier curves with dichotomized miRNAs at the median [Manier S. *et al*., 2017]. These data indicate that specific miRNAs can be critical in defining worse prognosis in patients with newly diagnosed MM. Nevertheless, to establish circulating exosomes as biomarkers, well-designed clinical trials are required. So far, there is no trial registered that is relevant to hematology and investigates circulating exosomes as a predictive marker in hematological malignancies. Exosomes are currently viewed as tumor cell surrogates or 'liquid biopsies' and as a promising non-invasive metrics of cancer. Exosomes might emerge as the most informative non-invasive predictors of cancer outcome orresponse to therapy.

EXOSOME-BASED HEMATOLOGIC MALIGNANCIESTHERAPEUTICS

Exosome-based therapies serve as attractive strategy against hematologic malignancies and solid tumors. Exosome-based delivery methods have been tested in the clinic successfully and were found to be well tolerated in patients. Being autologously generated within the host, they can be engineered to carry drugs or target proteins without invoking immunogenic response. A number of different strategies have been applied to harness the potential of these exosomes. Nano injections of RNAi in dendritic-derived exosomes allowed delivery to the brain without invoking immune response. These findings were confirmed when Gurwitz *et al.* showed that siRNAs can be delivered across the blood brain barrier in a mouse model using systemic injections of exosomes, (Gurwitz, 2016).

In another study, tumor-derived exosome-pulsed DCs, tumor-derived exosomes, and exosomes isolated from malignant ascites all have been investigated for their ability to elicit antitumor immune response in patients, (Liu *et al*., 2018). Likewise, their efficacy on exosome release or against exosomes has also been tested in different laboratories independently. These are some of the studies highlighting the benefit of applying nanobased assays in the design of exosomes drug therapies for cancer. However, its clinical utility needs to be tested in future studies. At present, there are several clinical studies that utilize an exosome-based regimen for solid tumors, (Que *et al*., 2016), but there is no clinical trial about exosome-based agents against hematologic malignancies (ClinicalTrials.gov Website keyword search exosome). These studies clearly point to an unexplored area of research where researchers can find answers to some of the unexplained mechanisms attributed to the multifaceted natural agents against hematologic malignancies. Altogether, exosomes have multiple potential clinical uses including the development of vaccines for

targeting tumors; also, tumor-derived exosomes may be useful as surrogate endpoints in evaluating therapeutic and preventive approaches to hematological tumors.

CONCLUSIONS

Success in diagnosis and treatment of complex hematological malignancies is dependent on our full understanding of the intricacies of interactions between different components within tumors. On the one hand, a number of studies showed exosomes are emerging as major players in inter- and intracellular communications. Exosomes have been shown to secrete diverse biological molecules, which are in the context of tumor cells survival, metastasis, drug resistance and immune evasion. On the other hand, tumor relevant exosomes play ~~the~~ important role in the areas of diagnostics and drug therapy, regenerative medicine, and vaccines. Taken together, technology and biology will inevitably pave the way for the future use of exosomes analysis in many preclinical research and clinical applications.

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Clinical uses and toxicity of Ergot, *Claviceps purpurea* An evidence-based comprehensive retrospective review (2003-2017)

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ABSTRACT

Ergot alkaloids produced by the fungus *Claviceps* parasitizing on cereals presents a high toxicity risk for animals and humans due to presence of its alkaloid content. Ergot-alkaloid toxicity occurs *via* their medicinal use however human poisoning from ergot plant is rare. The aim of this review is to determine the toxicity of ergot plant or ergot-amine derivatives in humans. Databases such as Google scholar, PubMed, Scopus, Web of Science alongwith related books and theses as well as the library resources from Imam Abdulrahman Bin Faisal University were used whereas the Key words searched included; Ergot, Egotism, Ergotamine poisoning, Ergotamine interaction with HIV therapy, Ergotamine overdose, and report cases of ergotism. The relevant literature search suggests many toxicity cases and side effects associated with the use of ergot-alkaloids. More commonly it develops a condition known as ergotism i.e. a disease or toxic condition produced by eating ergot grains or rye or chronic use of ergot drug. The characteristic

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features of ergotism consist of painful and cold extremities. Furthermore, a notable and severe interaction of ergot-alkaloids was found in HIV patients using ergotamine and antiretroviral drugs together. Ergotamine which is one of ergot preparation has poisonous effect when taken in over dose and it interacts with antiretroviral drugs also.

KEY WORDS: EGOTISM; MIGRAINE HEADACHE; POSTPARTUM HEMORRHAGE; ARTERIAL SPASM; CYANOSIS

INTRODUCTION

The ergot fungus (*Claviceps purpurea*) is family of secondary metabolites and is a common seed pathogen of grasses and cereals. Ergot is a parasitic fungus that belongs to the genus Claviceps and forms dark sclerotia on various grasses and grains. After maturing, the sclerotia fall to the ground and remain partially buried for the resting stage of ergot formation. This period occurs during cold or winter weather, (Ehrlich *et al*., 2013). Ergot plant has medicinal uses to treat some diseases; even though, Ergot contain alkaloids which can cause severe toxicity in mammals when ingested and thus the fungal infection might provide protection for the host plant against mammalian herbivores, (Panaccione *et al*., 2012). Ergot alkaloids are natural products having nitrogen indole alkaloids. There are three group of ergot; I. peptides (ergopeptines), II. Clavines and III. Lysergic acid amides, (Gerhards *et al*., 2014).

There are more than fourty known ergot and usually six of them may cause toxicity by interaction with neurotransmitters. These are ergotamine, ergocornine, ergocryptine, ergocristine, ergosine and ergometrine, (Mulac et al., 2011). Ergot alkaloids have pharmacological and toxicological effect on several receptor systems in the human body. Ergot become activated in the body to some receptors and show cytotoxic affects and induces apoptosis in human primary cells, (Mulac et al., 2013).

Ergotism occurs when either the fungus is ingested via the contamination of grain, for example, rye or by the medicinal use of drugs derived from ergotamine compounds. Ergot toxicity called Ergotism previously known as Holy Fire, in some cases may lead to death, (Floss, 1976). There are two forms of Ergotism; gangrenous and convulsive and both can occur in the same individual. Gangrenous named as Saint Anthony's, usually characterized by pain in one limb particularly the calf and leg, inflamed, swollen, and violet hand and foot whereas in severe cases the limb will separate from the knee with hemorrhage and may need a knee amputation. The other form of ergotism i.e. convulsive form is accompanied with heaviness and numbness in limbs with paresthesia well as diarrhea without vomiting. In humans, ergot is used pharmacologically to inhibit lactation, postpartum hemorrhage, and induce labor, (Lee and Coll, 2009). Previously ergot was used in the treatment of Parkinsonism and other endocrine and neurological disorders, (Tesh, 2015).

In addition, ergot induces cytotoxic and apoptotic effect shows anti-cancer effect and thus used as a cancer therapy. In a study on two cancer lines (Hep-G2 and HT-29) ergot showed a high grade apoptotic effect, (Mulac *et al*., 2013). Furthermore, ergot alkaloids have an important widespread use in migraine headaches and 5-hydroxytryptamine (5-HT) receptors acts as binding site for these ergot derivatives such as Ergotamine, Dihydroergotamine, and Methysergide. They work as agonist of 5-HT1B, 5-HT1D, and 5-HT1F receptors to treat for treatment as well as prophylaxis for migraine headache with a high efficacy, (Dholf *et al*., 2012). Though in human, ergot complications i.e. ergotism appears rarely with symptoms such as tachycardia, hypertension and arterial spasm, still it is important to be taken care as ergot acts as a strong vasoconstrictor and may cause severe adverse effects at cardiac and limbs level, (Adam *et al*., 2014).

Current review aims to highlight the more important and recently reported cases regarding ergotism in humans with major symptoms observed, as associated with the use of ergot-alkaloids. The literature will be searched to extract out the factors behind ergotism and to report the possible measure in order to tackle such reasons. The study owes importance as it will compare the therapeutic and toxic profile for ergot and to conclude an overall scenario of how to use ergot-alkaloids for therapeutic purposes and to avoid ergotism.

MATERIAL AND METHODS

Various databases searched were: Google scholar, Science direct, Research gate, Web of Science, PubMed, Science Finder, Scopus and Journals such as; Journal of Ethnopharmacology, Frontier in Ethnopharmacology, Thieme journals, Clinical Toxicology journal, Journal of Agricultural and Food Chemistry, Journals of Toxicology, Canadian Medical Association Journal, JSM foot and ankle journal, Toxicology and Industrial Health (SAGE journals) and The Royal College of Physician of Edinburgh were searched. In addition, books and theses and online as well as hard resources from library of Imam Abdulrahman Bin Faisal University Damam, Saudi Arabia was also searched.

Key words were also searched:

Ergot, Ergot alkaloids, reported case of Ergot toxicity, adverse effects of ergot; ergotism, ergotamine toxicity,

clinical cases observed for ergot alkaloids, uses of ergot alkaloids, interactions of ergot alkaloids.

The literature was searched for retrospective fifteen years i.e. 2003-2017. All the clinical cases regarding ergot toxicity or ergotism were gathered and filtered as per the inclusion and exclusion criteria as mentioned below;

Inclusion criteria

The clinical cases reported in humans associated with the use of ergot or ergot alkaloids in any of its form used, were included. The toxicity resulted due to overdose, long term use as well as any adverse effect and drug interaction of ergot and ergot derivatives with HIV drugs were also included in the study.

Exclusion criteria

Clinical cases regarding ergot toxicity, reported in animals or *in vivo* studies (cell lines) as well as *in vitro* studies were excluded from the study. Any clinical report with minor side effects or adverse effects was also excluded. Similarly, any interaction with conventional medicine other than ergotism was excluded from the study.

Search result

The number of clinical cases, after an in-depth and extensive review as well as the cases which met the inclusion/exclusion criteria was found to be ten. These cases are reported in detail in the forthcoming section of literature review;

LITERATURE REVIEW

The ten cases filtered as per eligibility criteria are explained in detail as below. These cases are reported here-in an ascending year wise order;

A. Clinical case reported in 2003

i. Overdose of Ergot preparation

A 36-years old man was diagnosed with HIV and lymphoma eight years ago and he was taking triple antiviral therapy. He developed bilateral claudication and foot cyanosis. History revealed that the patient was taking 2 mg of ergotamine tartrate due to migraine headache. The overdose of ergot-alkaloid developed paresthesia, and coolness in feet without pain. The left foot was cyanotic and he was administered with heparin IV and oral nifedipine. The symptoms resolved within one week of the therapy, (Badwin and Ceraldi, 2003).

B. Clinical case reported in 2005

ii. Ergot toxicity in neonate

An infant born at 41 weeks gestation period was accidentally administered with methylergonovine (I.M.) instead of naloxone for respiratory depression. Soon after administration he suffered from capillary refill, low oxygen saturation, his hand and feet became warm and pink and after sometime his color became grey with development of hypercarbia (partial pressure of carbon dioxide). The infant was put on mechanical ventilation and treated with nitroprusside infusion. The condition recovered with 10 days of hospital treatment, (Bangh *et al*., 2005).

C. Clinical case reported in 2009

iii. Ergotamine with caffeine

A 28-year old patient reported to hospital with pain in both legs. Upon history it was revealed that the patient used ergotamine tartrate (1 mg) with caffeine (100 mg) for 3 days, due to a bi temporal headache a week ago. Thus he developed the symptoms of severe leg pains, especially below the ankle with cold and purple legs. Hands were also cold but without any pain. The patient was treated with I.V crystalline fluids and nitroprusside as well as oral nifedipine (every 8 h). Papaverine was also administered as the patient had arterial spasm whereas Enoxaparine was administered in order to inhibit any thrombus formation, (Musikatavorn *et al*., 2009).

D. Clinical case reported in 2009

iv. Ergotamine with Anti-HIV

A 31-year female patient on anti-HIV therapy used ergotamine for migraine headache. Soon after she start to complain about pain in lower extremity and her both legs were cold particularly the left leg. In addition, a palpable popliteal artery with peripheral pulses was also observed. This condition is termed as Ergotism. The sign and symptoms resolved gradually with the use of Nifedipine (30 mg) and Enoxiparine was used as treatment for Ergotism, (Cagatay *et al*., 2009).

E. Clinical case reported in 2010

v. Ergotamine used with clarithromycin (Antibiotic)

An eighteen year ole female patient reported with a pain in emergency which started 2 days ago. The symptoms she presented were; cool cyanotic lower extremities, tender on palpitation, pain at rest, a thin iliac to popliteal region and blood flow with increased velocity. The history revealed that the patient was using ergotamine since 3 year for migraine whereas at the same time she used clarithromycin for upper respiratory tract infection. Heparin was administered in order to prevent possible thrombotic complications and infusion of bupivacaine was given. The symptoms resolved within a month, (Demir *et al*., 2010).

F. Clinical case reported in 2010

vi. Ergotamine interaction with anti-HIV drugs produces paresthesia

A 29-year old patient using antiretroviral therapy for HIV traveled Egypt and developed a severe migraine. A local physician prescribed him ergotamine tartrate for two weeks. Soon after its use, he developed sever pain in left upper extremities, pale and cool wooden hand with paresthesia, absent arterial pulse and axillary artery stenosis, and he was facing difficulty in writing. Heparin was started as therapy to inhibit any coagulation. Prostacyclin IV and topical Nitroglycerine was used as therapy. The symptoms resolved within six days, (Frohlich *et al.*, 2010).

G. Clinical case reported in 2012

vii. Neonate and methylergonovine

A female neonate, 10 minutes after her delivery, was given methylergonovine instead of vitamin-K. after 30 minutes, she developed respiratory depression with cyanosis in extremities. Naloxone IM was administered to recover the symptoms, (Sullivan *et al.*, 2012).

H. Clinical cases reported in 2014

viii. Ergotamine with azithromycin (Antibiotic)

A 35-year old women reported with severe pain and pallor in left foot, into emergency room. She was using ergotamine since long time due to migraine, and in addition she used azithromycin for four days (as she was heavy smoker). Upon examination, vasospasm and acute arterial embolism was observed due to interaction between azithromycin and ergotamine. For treatment purpose, ergotamine was stopped and Heparin was administered for a week. The symptoms resolved gradually,(Adam *et al.*, 2014).

I. Clinical cases reported in 2016

ix. Ergotamine use with caffeine

A 33-year old women presented with critical limb ischemia, foot camping pain and a decreased walking distance for 36 hours. Two days ago she used ergotamine tartrate (1 mg) and caffeine (100 mg) for migraine headache. She was treated with vasodilator (PG-E1 I.V.), pentoxifylline, heparin and sildenafil for every 8 hours. The patient recovered partially within 4 days, however for full recovery she was further prescribed with aspirin, sildenafil and cilostazol, (Eduardo *et al.*, 2016). *Ergotamine overdose develops cerebral ergotism*

A 49-year old patient with a history of HIV infection treated with ritonavir used ergotamine (3g) for migraine. He developed cerebral ergotism with an ischemic attack after 20 minutes. The Cervical Doppler ultrasound revealed a narrowing in both internal carotid arteries. The treatment plan consist of; stopping ergotamine, and changing the antiretroviral regimen, aspirin (325 mg) was administered and bed rest suggested. The patient became asymptomatic after a week. (Gaye-Saavedra *et al.*, 2016) The cases reported for Ergot are presented in detail in Table 1, as below;

DISCUSSION

Ergot, with the most prominent member in group i.e. *Claviceps purpurea,* grows on rye and other grasses. It is considered as a poisonous plant that produce ergotism in humans and sever toxicity in mammals. The improper use or overdose of ergot may lead to various severe complications, discussed as below.

Ergot develops psychological effects; convulsive, spasmodic or nervous ergotism i.e. chronic serious stage of ergotism called "convulsive stage", can occur due to patchy damage of mid brain and cerebral hemisphere which leads to degeneration in the posterior column of spinal cord. The patient in such cases suffers from opisthotonos strong muscle spasm, tonic or clonic spasm of limbs, battened tongue, dilated pupils, mania, dementia, glaucoma, and delirium. In addition, the patient may develop status epilepticus/multiple convulsions with less or lack of sleep and finally coma and death, (Lee and Coll, 2009).

Ergonovine evoke coronary artery spasm (CAS); earlier, ergonovine (i.v.) was considered as a useful test for CAD diagnosis however, recently it has been reported that it may provoke variant angina in patients. It effects vascular smooth muscles via alpha adrenergic agonist activity and leads to vasoconstriction. Similarly, in CNS it blocks the baroreceptors and prevents response of reflex bradycardia. Thus it is considered an unsafe drug in CAD patients because ergonovine may develop severe hypertension, myocardial infarction, seizures, intracerebral hemorrhage and death, (Curry and Pepine, 1977).

Acute coronary syndrome with myocardial bridging; Ergotamine may induce vasoconstriction in coronary, cerebral and pulmonary arteries and leads to change in ECG pattern. A case of ergotamine overdose was reported in 2003, for a 48-year aged women with migraine headache, whereby she developed recurrent chest pain with a complaint since last 10 days. The ECG showed T-wave inversion and elevated ST-segment. This shows that ergotamine may cause serious cardiac adverse effects such as; arrhythmias, coronary vasospasm and death, (Shimony *et al.*, 2006).

Induces serotonin syndrome; it can be a mild to life-threatening syndrome. Dihydroergotamine, binds to serotonin receptors in the dorsal horn of spinal cord, result in serotonergic hyper-stimulation and may

Table 1. Clinical cases reported for ergot

Case No.	Age	Gender	Clinical manifestation	Cause	Treatment plan
1	36	Male	-Paresthesia and coldness of left foot -Loss of sensation	Ergotamine tartrate for migraine	-Heparin-IV -Nifedipine orally
2	41 weeks	Male infant	-Warm pink hands and feet -Low oxygen saturation -Hypercarbia-Oliguria	- Methylergonovine IM	- Nitroprusside infusion -Furosemide
3	28	Male	-Painful ,cold, and purple left legs especially below ankle -Arteries spasm	-Ergotamine tartrate for bi temporal headache	-Crystalloid fluids(IV) -Sodium nitroprusside (IV) -Nifedipine orally -Papaverine for arteries spasm -Enoxaparin to prevent thrombus formation
4	31	Female	-Pain, cold lower left extremity -Palpable popliteal artery	-Ergotamine for migraine +D-DI with anti-HIV	-Nifedipine -Enoxaparin
5	18	Female	-Cool cyanotic lower extremities -Tender on palpitation -Thin arteries with increased blood flow velocity	-Ergotamine for migraine	-Heparin -Infusion of bupivacaine
6	29	Male	-Painful cold left upper extremities -Pale wooden hands Paresthesia -Writing difficulties	Ergotamine tartrate for migraine for 2 weeks + D-DI with anti-HIV	-Heparin -Prostacyclin IV -Nitroglycerin(dermal application)
7	10 min	Female	-Respiratory depression -Cyanosis in her extremities	Unintentional use of methylergonovine	Naloxone IM
8	35	Female	-Pain and pallor in the left foot -Vasospasm and acute arterial embolism	Ergotamine for migraine	Heparin for 7 days
9	33	Female	-Critical limbs ischemia -Foot camping pain -Decreased walking distance	Ergotamine tartrate for migraine+ D-DI with ritonavir	- Prostaglandin E1(IV) - Pentoxifylline - heparin - sildenafil every(8h)
10	49	Male	-Cerebral ergotism -Ischemia	Ergotamine overdose +D-DI with ritonavir	325mg aspirin+ bed rest

develop "convulsive ergotism" characterized with symptoms of behavioral changes and mental disturbances, agitation, sweating, fever, and hallucination etc. (Eadie, 2003).

Ergotamine and its interaction with macrolide antibiotics; the combination of ergotamine with erythromycin is considered and reported as a dangerous combination. The reason behind is; macrolides have hepatic circulation with ergotamine whereby it causes severe vascular spasm and transient renal ischemia. And this is due to macrolide inhibition of cytochrome P-450 metabolism leading to an increase serum ergotamine concentration. (Ghali *et al*., 1993) A clinical case reported in 2010 about a woman with sore throat and migraine. She was prescribed with erythromycin (500 mg) and ergotamine (100 mg) and soon after the combined use of these drugs she developed pain and paresthesia in both feet as well as numbness in hands. (Tseng *et al*., 2010) Though ergot may have unwanted side effects or adverse effects as observed above, however it is also a well-known fact that ergot possess potential advantages as discussed below.

Bromocriptine as treatment of AML: acute amyloid leukemia is a type of cancer that affect the blood and bone marrow. Bromocriptine is a hydrogenated ergot derivatives with dopamine agonist activity, a novel therapy as Anti-AML, induce apoptosis for AML cells. (Lara-Castillo *et al*., 2016).

Bromocriptine as a treatment of breast cancer: there is a role of dopamine in development of cancer whereas bromocriptine via down regulation of prolactin, result in decrease and suppress the proliferation of MCT-7 cells and induce apoptosis. It also shows effectiveness in idiopathic granulomatous mastitis (IGM) associated with hyperprolactinemia. (Majid *et al*., 2015).

Ergot still the best as compared to triptans: ergot is still a drug of choice in migraine. It has more good activity as compared to triptans as a powerful vasoconstrictor because it works longer and better for prolong attacks than some triptans. Ergotamine is still very useful and is used in many countries to relief migraine headache. Rectal ergotamine reported higher efficacy as compared to triptans with rectal ergotamine activity of (73%), while triptan rectally was (63%). (Marcelo *et al*., 2003).

Reduce postpartum hemorrhage: Ergotamine IM/IV, as prophylactic agent, shows a good reduction in postpartum hemorrhage when coadminstered with analgesic to reduce the pain. An optimum result predicted with intramuscular injection of ergotamine oxytocin combination called (Syntometrine) at the end of second stage of labor. (Liabsuetrakul, 2007).

Ergot toxicity is due to cytochrome P-450: Ergotamine is metabolized via CYP enzyme and interaction with enzyme inhibitors drugs can increase its level in the body which will lead to toxicity such as in the case of antiviral and antibacterial drugs. Grapefruit also has been reported to increase the level of ergotamine, thus it shows that toxicity as resulted may not be due to ergotamine itself, but due to interaction with drugs inhibiting the enzymes. (Badwin and Ceraldi, 2003) Current review consist of clinical cases reported due to use of ergot. It was observed that the misuse or use without proper medical guidance may result a condition known as ergotism. The major symptoms for ergotism includes; vasospasm, arterial embolism, pain and coldness in feet especially the left one, respiratory depression particularly in neonates, paresthesia and decreased walking distance. The treatment applied for these conditions mostly included vasodilators and Heparin and almost all of the cases observed were due to ergotamine overdose or drug-drug interaction with protease inhibitors. In few of the cases an interaction among ergot alkaloids and other drugs, especially antivirals and azithromycin, was observed when they are administered together. Furthermore, in most of the cases it affected elder people i.e. age of 30 or likewise, however it can affect infants when it's given by medical mistake. Regarding gender it effects both female and male equally however it was observed that patients with HIV disease are more prone to ergotism due to severe interaction. Two cases were observed where toxicity of ergot resulted due to overdose of ergotamine.

Plants remains the well-known sources of treating many ailments mostly working as antioxidant, antidiabetic and antiglycation, (Ahmad *et al*., 2013 & 2014) and the people mostly possesses sound knowledge regarding the use and treatment of most of the plants (Ahmad *et al*., 2017).

However, in most of the cases the poisoning or toxicity cases are not known to common peoples. Herein, we mentioned ten poisoning cases for ergot where the general and common symptoms observed in elder patients, due to toxicity were; painful and coldness lower extremity especially the left foot, ischemia and purple legs whereas, the symptoms of affected infants were; respiratory depression and pinkish skin. In some cases the lab tests showed normal hemoglobin, white blood cells with a normal temperature (37-37.7C) measured orally. The common treatment used in such toxicity cases was; aspirin, low molecular heparin given IV, and nifedipine orally.

CONCLUSION

Finally, ergot plant has medicinal uses as well as toxic effects. Current review has found poisoning cases which were mostly due to overdose, mistakenly administered or interaction with anti-HIV drugs for ergot alkaloids. Any disease condition using ergot with proper dose, time and under sound medical supervision whereby the

interaction with other drugs is taken under consideration, may result a proper therapeutic outcome for ergot.

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Formation of the control signals based on application of the neural network approaches in spine rehabilitation systems

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ABSTRACT

The article substantiates the necessity of correction of control signals depending on the state of the spine and the movements performed. The generalized structural scheme of the control unit of executive mechanisms in spine rehabilitation systems is considered. As a result of the operation of the control unit, motor exercises are corrected in rehabilitation techniques based on the results of modeling the permissible degree of flexure of the spine. An example of patterns of motor actions is given and a generalized model of motion patterns is described.

KEY WORDS: DIAGNOSTIC, REHABILITATION, NERVOUS SYSTEM, GONIOMETRY, MUSCULOSKELETAL SYSTEM, NEURAL NETWORK

INTRODUCTION

The efficiency of modern technical means makes it possible to implement algorithms for real- time processing large amounts information. It is lead to increase the efficiency and quality of medical systems, in particular, rehabilitation systems of the spine. The complexity of developing of this class systems is associated with a greater risk of harm to the health of the patient due to an incorrect diagnosis. This risk also includes erroneous decisions of the rehabilitation system that are incompatible with the life of the patient. The need for adaptation to the various physiological parameters of the patient taking into account of injuries to the spine causes even greater difficulties, (Sobolev *et al.*, 2017 Kulik, 2017).

The aim of the present work is to improve the quality of management of rehabilitation exoskeletons due to the use of neural network algorithms for estimating the permissible degree of flexure of the yesterday.

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THE PROBLEM OF MANAGEMENT OF THE REHABILITATION SYSTEMS

Currently, the functionality of existing rehabilitation systems is insufficient for patients with spine pathologies. This is due, first of all, to their limited application in conditions of low mobility of the patient (fractures, gunshot wounds, etc.) or the lack of a priori information on the patient's permissible movements (during rehabilitation) without causing additional harm to his health. However, the process of rehabilitation is accelerated if the correct load on the pathological parts of the spine is calculated (Yezhov *et al.*, 2013; Tuktamyshev & Bezmaternykh, 2014; Vengerova & Solovyova, 2008; Zubareva, 2011, Maksimova, 2012; Kulik, 2017, Sobolev *et al.*, 2017).

The control signals for the rehabilitation exoskeleton are formed on the basis of the patient's desired movements and are limited by the physiological parameters and state of the patient.

Arbitrary movements of the patient are formed by the exoskeleton on the basis of the recorded nerve (electroencephalography), muscle (electromyography). Involuntary movements of the patient are formed by the mechanical (strain gage) signals of the exoskeleton at different stages of motor processes in various (informative) areas of the patient's body, (Grecheneva *et al.*, 2017).

Problems in recording arbitrary movements of the patient are the error of the measuring path, the quality of recognition of informative signals and pathology of the human neuromuscular system.

So, for example, all movements of the musculoskeletal system of a human without pathologies begin in the central nervous system, namely in the motor zone of the cerebral cortex. The generated electrical signals of movement (motion impulses) from the brain through the spinal cord are transmitted to the peripheral nervous system along those nerve fibers (motor neurons) that must cause the necessary contractions of the muscular system, (Sobolev *et al.*, 2017).

Motor neurons have feedbacks, which receive information from muscle fibers, receptors and other sensory receptors, in order to further coordinate movement and prevent muscle damage. Since the moment of formation of an impulse in the cerebral cortex before the movement (contraction or relaxation of the muscles), some time passes, individual intervals of which are described in (Sinitskaya & Gribanov, 2014; Zakharova *et al.*, 2012; Grecheneva *et al.*, 2017).

In general, the movement (especially arbitrary) is the result of complex neuropsychophysiological processes in which a plan of motion or reaction to stimuli is formed, and its constant correction occurs throughout the entire movement. In addition to the motor zone of the cerebral cortex, other areas of the brain are involved: the posterior parietal cortex, the limbic system, the cerebellum, the frontal cortex, etc. (Sinitskaya & Gribanov, 2014).

When processing and analyzing the signals of motor neuron activity, attention should be paid to the fact that useful signals, although cyclic, are not stationary. In addition, the distribution of the noise component of the signals is not normal (Zakharova *et al.*, 2012).

Functional changes of any part of the path from the place of formation of motor signals to the muscle cause changes in the parameters of motion of the involved kinematic pairs and the musculoskeletal system as a whole. Figure 1 shows the averaged electromyograms obtained (Fig. 2a and 2b) and the dynamics of the deviation angle from the axis of the spine (Fig. 2c and 2d) in the state of rest of a healthy person (Fig. 2a and 2c) and a person with a tremor of the back muscles (Fig. 2b and 2d) (Butukhanov, 2009).

Deviations in the electrophysiological signals involved in the locomotion activity of the musculoskeletal system, from normal values for healthy people manifest themselves in amplitude, phase, shape, and other characteristics of the signals and depend on the different concentration of the attention, the accuracy etc. (Voznesenskaya, 2006; Doronin & Doronina, 2010; Rakhmilevich *et al.*, 2012; Efimov, 2012, Zakharova & Shemirova, 2016; Shchenyavskaya & Zakharova, 2015; Zakharova *et al.*, 2016).

The need for high accuracy of recording of the patient movements is due to possible damage to nerve fibers and the nervous system as a whole. When a nerve tissue is damaged, a number of processes occur successively, leading to the death of damaged nerve cells and the subsequent death of intact ones. According to modern ideas, the main factors leading to the destruction of nerve cells are a violation of microcirculation, hypoxia and ischemia. There is a link between the degree of neuronal damage and the change in the level of the constant potential and the membrane potential of neurons (Sufianova and Shapkin 2014; Shanitsin *et al.*, 2013).

Damage to the nervous tissues of the spinal cord changes the frequency and amplitude of the spinal cord signals and depends on the amount of pressure (compression) and the degree of damage to the nerve fiber. Relying on the works on a dependence of the amplitude of the electrospinogram on the subdural pressure can be described in accordance with Table 1.

Thus, damage to the spinal cord causes an increase in spontaneous electrical activity, and in case of significant damage, further decrease in spontaneous electrical activity. In this case, the frequency characteristics of the activity of the spinal cord correspond to the frequency characteristics of activity of the cerebral cortex, but with

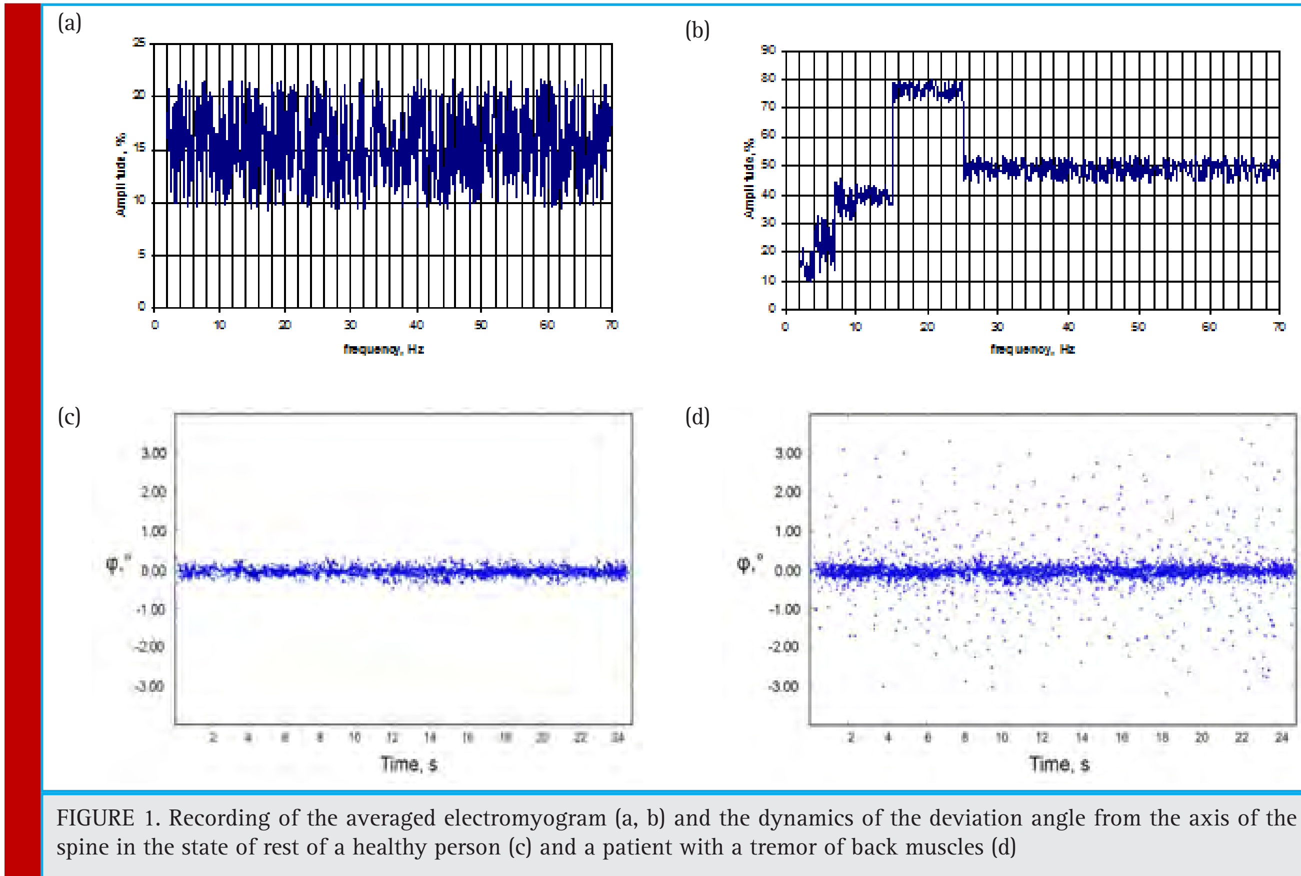

FIGURE 1. Recording of the averaged electromyogram (a, b) and the dynamics of the deviation angle from the axis of the spine in the state of rest of a healthy person (c) and a patient with a tremor of back muscles (d)

smaller amplitude (in the spinal cord). A rupture of the spinal cord increases the level of the constant potential and lowers the values on the electro-spinogram of segments lying below the trauma, and leads to an increase in electrophysiological changes as the distance decreases from damage.

Segments lying above the damage zone are characterized by a decrease in the level of the constant potential and the total amplitude on the electrospinograms, a decrease in the magnitude of electrophysiological deviations. With pressure on the spinal cord, the level of the constant potential shifts and the amplitude decreases on the electrospinograms. The degree of violations in signals decreases is removed from the site of pressure. When the pressure on the spinal cord decreases, repolarization occurs and the amplitude increases again on the electrospinograms. A complete restoration of the level of constant potential does not occur. Thus, the complex processing of the values of the level of the constant potential and electric activity of the spinal cord makes it possible to evaluate the electrophysiological violations and functional changes in the spinal cord both in the injury zone and in neighboring areas (Kulik, 2017).

Thus, the formation of control signals for the rehabilitation exoskeleton is determined not only by the chosen recovery technique, but also by the patient's vertebral state (vertebral, interarticulate fluid, interarticulate cartilage and neural fiber regions).

CONTROL UNIT FOR REHABILITATION EXOSKELETON

The task of the control unit for the rehabilitation exoskeleton is to generate control signals for the actuators (Figure 2).

The control unit consists of two functional parts: a forecast generation unit and a decision block. The forecasting unit evaluates the location, extent and likelihood of damage to the bony, cartilaginous and nerve tissues of the spine. The prognostic estimation is formed on the

Table 1. Dependence of the amplitude of the electrospinogram from subdural pressure

Pressure, mm. gt;	183	250	300	350	400	450	500	550	560
Amplitude, μV	31±9	40±10	52±12	54,5±15,5	64±18	78±20	82,5±15,5	73±15	68±15

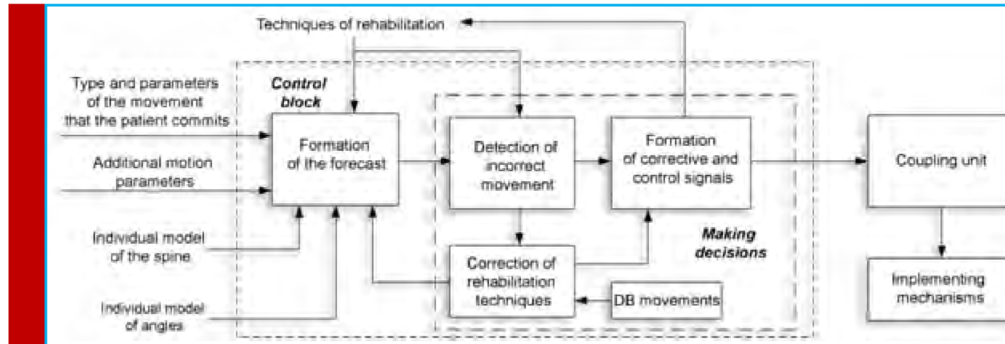

FIGURE 2. The control unit for rehabilitation exoskeleton

basis of the information angular model of the patient, characterizing the permissible deviations in patient movements, the individual spine model describing the geometric and spatial parameters of the main parts of the spine, the data on the movement (type, speed, angles and EMG and EEG data). The performed movements are performed in accordance with the rehabilitation technique. The algorithm of the control unit is shown in Figure 3.

A lot of work has been devoted to the main dependencies of the behavior of the musculoskeletal system, joints and their connecting components, pain sensations and thresholds of perception of pain, for example (Pezhovic *et al.*, 2003; Pinchuk *et al.*, 2008; Shilko & Ermakov, 2008; Suslov *et al.*, 2008; Babchina *et al.*, 2017; Grecheneva *et al.*, 2016; Grecheneva *et al.*, 2017). Formation of prognostic estimates of damage to the spinal sections during motion on the basis of individual models and parameters of movements are given in (Dorofeev *et al.*, 2017).

The vector of predictive estimates for each type of tissue is described by the vector F={L(X,Y,Z), P(X,Y,Z)}, where **L** is the three-dimensional vector for estimating the degree of damage, and **P** is the damage probability. The change in the projection vector in time **F(t)** is used in conjunction with the vector M**(t)**, describing the rehabilitation technique (rehabilitation exercise). The vector M(t)={K(t), C(t)} characterizes the space-time change in

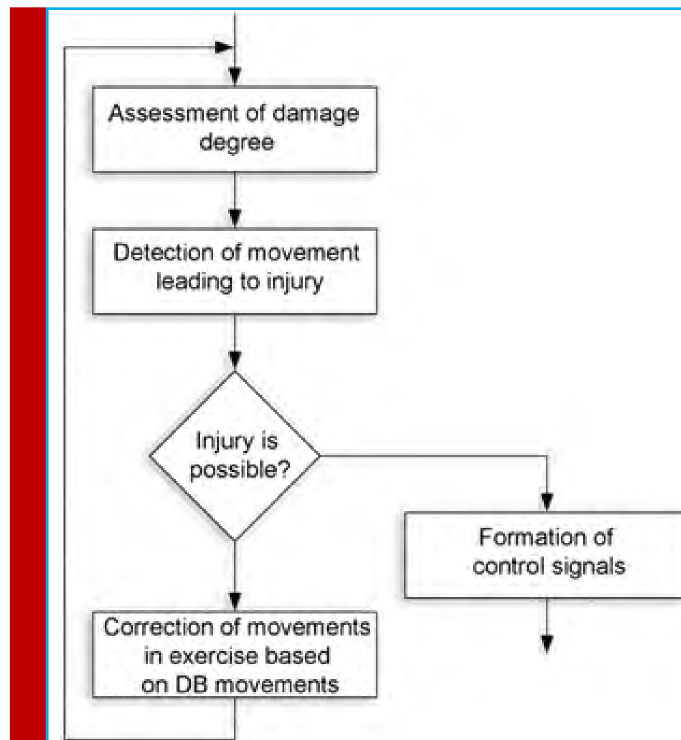

FIGURE 3. The algorithm of the control unit

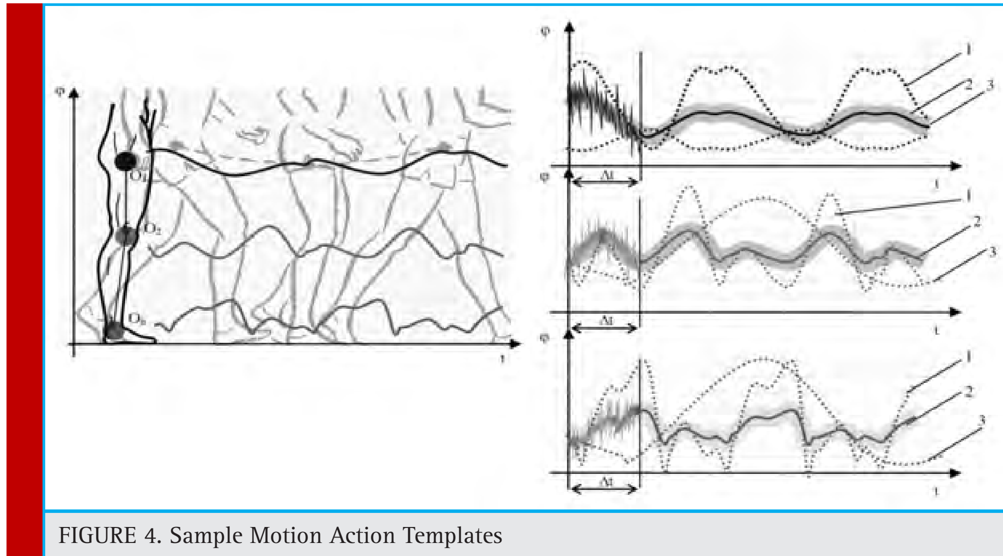

FIGURE 4. Sample Motion Action Templates

the position of the kinematic pairs of the spine **K(t)** and the space-time characteristics of the motion (tempo, frequency, etc.) *C***(t)**.

Classification of movements occurs on the basis of pre-formed patterns of motor actions (Figure 4). The motion pattern can be described by the vector

$$\vec{T}(t) = \{\vec{T}_D(t), \vec{Ib}(t), \vec{Un}(t), \vec{P}st, \vec{P}pt\} \quad (3)$$

where $\vec{T}_D(t)$ is the vector of spatial change in the position of the kinematic pairs; $\vec{Ib}(t)$, $\vec{Un}(t)$ are the vectors describing the change in electrophysiological parameters during the time of motion (some examples are presented in [16]); $\vec{P}st$ is the vector describing the spectral-temporal characteristics (frequency, power spectra, etc.) of patterns of goniometric and electrophysiological signals; $\vec{P}pt$ is the vector describing the space-time characteristics of the motion (tempo, amplitude, speed, acceleration, etc.).

Motion patterns are stored in a database, supplemented for individual characteristics and various pathologies. Database updates are necessary for automatic learning and retraining of the neural network.

When implementing the system of direct control of the exoskeleton, it is necessary to have information about the magnitude and position of the goniometric vectors and vectors of the stator and exoskeleton rotor linkage, which are measured by means of various sensors. Unlike vector systems, the direct torque control system uses only current and voltage sensors and does not require the use of a speed sensor. However, accurate estimation of the position of the flux-linkage vector of each of the exoskeleton servo drives is problematic, therefore, state observers are often used to determine the flux linkage. In the case of a medical rehabilitation exoskeleton, the patient's angular model is an observer of the state, the input of which is measured goniometric data, the components of the servo vector of the state, and an output of the general state vector is output. As an observer, it is proposed to use an artificial neural network (Fig. 5).

It is assumed that combining the method of direct torque control and neural network technologies will sig-

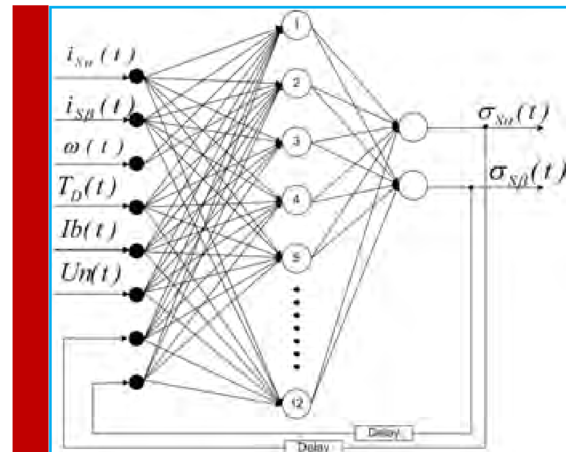

FIGURE 5. The structure of the neural network. Input signals of such a network are stator current signals $i_{S\alpha}$ $i_{S\beta}$ of the i-node of the exoskeleton, ω the rotor frequency, $\vec{T}_D(t)$ is the vector of spatial change in the position of the kinematic pairs; $\vec{Ib}(t)$, $\vec{Un}(t)$ are the vectors describing the change in electrophysiological parameters during the time of motion and feedback c from the neural network output delayed by one step of training (Delay block), and output signals - signals that determine the mode of operation of the exoskeleton nodes $\sigma_{S\alpha}$, $\sigma_{S\alpha}$.

nificantly improve the quality of control of an asynchronous traction electric drive, as well as the robustness of the control system (resistance to changes in the parameters of the control object), thereby improving the quality of control and identification.

Simulation of the operation of the control unit was performed on the CT of patient data, which has a curvature of the cervical spine. When the head was tilted to an angle of more than 59 degrees on average, the patient experienced pain. In 87% of cases, the head inclinations were accompanied by a slight crunch in the cervical region.

Initial exercises for modeling the operation of the control unit included the inclination of the head by 90 degrees. As a result of the operation of the control unit, the initial exercises were adjusted, the maximum inclination of the head was 64 degrees (Figure 6).

CONCLUSION

Thus, the developed algorithms of the control unit allow to correct motor exercises in the rehabilitation technique for the physiological characteristics of the patient, and also do not allow the executive mechanisms to make movements dangerous to health. It should be noted that the permissible limits for the search for optimal exercises of the control unit are set by the expert and for automated work should be automatically determined from the CT data and the simulation results. These boundaries in the example under consideration were set rigidly, which was the reason for the discrepancy between the results of modeling and pain sensations of the patient being studied.

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Bio-prospecting fungal endophytes of high altitude medicinal plants for commercially imperative enzymes

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ABSTRACT

Endophytic fungi have been a focal point of research as repository of extreme chemical diversity. Isolation of fungal endophytes from medicinal plants has led to detection of plethora of novel agents encompassing bioactive potential. In the present work, *Withania sominifera, Ocimum basillicum* and Syzygium aromaticum from high altitude region were selected for bio-prospecting of fungal endophytes. 14 fungal endophytes were recovered from different parts of *Withania sominifera, Ocimum basillicum* and *Syzygium aromaticum*. Maximum fungal colonization was recovered from *Withania sominifera and Syzygium aromaticum* (42.8 %). In the preliminary screening for production of commercially important enzymes including protease, amylase, cellulose and asparaginase activity, all fungal endophytes of *Ocimum basillicum* exhibited potent activity. However, In case of proteolytic activity, #9SASTD exhibited maximum proteolytic potential. Maximum amylase and cellulase production was observed in #2SASTD and #14WSLF respectively. Interestingly, isolates form *Withania sominifera and Syzygium aromaticum* exhibited potent asparaginase activity with maximum potential in #22WSLD. Thus, the data clearly indicates the potential of high altitude medicinal plants as source of endophytic repository that can be taken as measure to prevent exploitation of endangered medicinal plants for commercial use. Further studies are warranted for characterization of the fungal isolates.

KEY WORDS: ASPARAGINASE, ENDOPHYTE, *OCIMUM BASILLICUM, SYZYGIUM AROMATICUM, WITHANIA SOMINIFERA*

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INTRODUCTION

Endophytic fungi comprise a group of heterogeneous fungi which live asymptomatically inside the plant tissues without showing any sign of their existence (Strobel and Daisy, 2003; Muller *et al*, 2016). Fungal endophytes are considered to be "Gold Mine" of novel bioactive compounds with immense therapeutic potential in the pharmaceutical sector. Various anticancer, anti-inflammatory, antioxidant, antimalarial drugs have been reported from fungal endophytes that are currently being exploited as control measure for various medical conditions such as hyperchlosteremia, Leukemia, renal failure, cardiovascular disorders (Gouda *et al.* 2016; Raviraja *et al.* 2006). Fungal endophytes are also proved to mimic the bioactive properties of the host plant due to the horizontal gene transfer (Strobel, 2002; Strobel *et al.* 2004; Jia *et al.* 2016; Venieraki *et al.* 2017; Huang *et al.* 2018). Potential of medicinal plants form high altitude region in pharmaceutical industry is undebatable. Over exploitation of medicinal plants has been a threat of increase in number of endangered plants. Hence, fungal endophytes inhabiting the plants with medicinal values are under exploitation by various groups of researchers in the process of finding a novel chemical scaffold with therapeutic potential (Kapoor and Saxena, 2016; Kapoor and Saxena, 2018).

The present study was oriented towards the exploration of fungal endophytes inhabiting *Withania sominifera*, *Syzygium aromaticum and Ocimum basillicum* of Uttarakhand region, India. The main rationale behind selection of these plants from Uttarakhand region lays within the fact that endophytic mycoflora of Uttarakhand region is very sparsely explored for bioactive entities.

MATERIALS AND METHODS

PLANT SAMPLE COLLECTION AND ISOLATION OF ENDOPHYTIC FUNGI

Healthy parts (stem and leaves) of medicinal plants viz. *Withania sominifera*, *Syzygium aromaticum* and *Ocimum basillicum* were collected from Kanhaiya Vihar region, Dehradun during winter season. The samples were transported to laboratory in sealed pouches and stored at 4˚C. For the isolation of endophytes, surface sterilization of stems and leaves were carried out by dipping in 0.1% sodium hypochlorite solution for 2-3 min followed by 70% Ethanol for 1 min and then subsequent washing in 30% Ethanol for 30-45 sec. The surface sterilized samples was cross sectioned into small pieces of 1-2 mm size aseptically and were inoculated on to pre sterilized Potato Dextrose Agar (PDA) plates. The plates were then incubated at 26±2ºC, 16h/8h light/dark condition for 8-10 days. The plates were regularly monitored for any fungal growth. The fungal hyphae emerging out of the segment was transferred to fresh PDA plate aseptically with the help of inoculation loop to obtain pure culture (Mitchell *et al.* 2008).

SECONDARY METABOLITE PRODUCTION

All the fungal endophytes were subjected to secondary metabolite production by following the method of Raviraja *et al.* 2006. Briefly describing, 5mm mycelial plug of 4-5 day old active culture was inoculated into 100 ml pre-sterilized Potato Dextrose Broth (PDB) followed by incubation at 26±2ºC, 120 rpm for 7-10 days. After the culmination of incubation period, the culture filtrate was separated from mycelial mass by filtration through Whatman filter paper No. 4 followed by centrifugation at 10,000 rpm for 10 min. The supernatant so obtained was stored at 4˚C till further use.

SCREENING OF ENZYMATIC ACTIVITIES

For the protease activity, 1 % skim milk agar plates were prepared and 5 mm wells were punched out by sterile cork borer. To each well, 30 µl of each culture filtrate was added followed by incubation at 37˚C degrees for 24 h. Non-inoculated PDB served as control. After incubation, a clear zone around the wells indicates the proteolytic activity. The zone diameter was measured and expressed as Mean ± SD.

(a) Cellulolytic activity:

Cellulase activity was assessed by preparing Modified Czepak Dox (MCD) agar plates supplemented with 1 % carboxymethyl cellulose (CMC). 30 µl of each culture filtrate was dispensed in 5 mm well prepared by sterile cork borer in MCD-CMC agar plates. The plates were incubated at 37˚ C for 18-24 h. Un-inoculated PDB served as control. After the incubation is over, the plates were flooded with aqueous congo red solution. After 15 min, appearance of yellow zone around the fungal colony indicated Cellulolytic activity (Lingappa *et al.* 1962). The zone diameter was measured and represented as Mean ± SD.

(b) Amylolytic activity:

Amylase activity was assessed by preparing the 1% starch agar plate followed by preparation of 5 mm well with the help of pre-sterilized cork borer. Briefly, 30 µl culture filtrate of each fungus was loaded into the wells followed by incubation at 37˚ C for 18-24 h. Un-inoculated PDB served as control. After the incubation, the plates were flooded with 1 % iodine solution. Appearance of clear zone around the well indicated amylolytic

activity (Hankin and Anagnostakis, 1975). The zone diameter was measured and represented as Mean ± SD.

(c) Asparaginase Activity assay:

Production of Asparaginase by the fungal endophytes was assessed by modified Ditch plate assay (Mahajan *et al.* 2013). Briefly, L- asparaginase-agar (2 %) plates containing phenol red (0.009 %) were prepared and each plate was divided into four quadrants followed by preparation of 5mm wells in each quadrant using sterilized cork borer. Further, 30 µl of culture filtrates of each fungal endophyte were loaded onto the premade wells in L-asparaginase agar plates. The plates were incubated at 37°C for 24h. After the culmination of incubation period, the plates were observed for the pink halo formation around the wells. The zone diameter was recorded and expressed as Mean ± SD.

RESULTS

A total of 14 fungal endophytes were recovered from different parts of *Withania sominifera*, *Ocimum basillicum* and *Syzygium aromaticum* (Table 1, Figure 1). Maximum fungal colonization was recovered from *Withania sominifera* and *Syzygium aromaticum*(42.8%) followed by *Ocimum basillicum*(14.2%). The host tissue of each plant sample exhibited a variation in colonization of the endophytic mycoflora. The maximum colonization of fungal endophytes was observed in leaf (57.1%) followed by stem (28.5%) and internal tissue/vascular tissue in the stem (14.2%). No endophyte was recovered from the stem and stem internal tissue of *Withania sominifera* and leaf of *Ocimum basillicum* (Table 2).

SCREENING OF BIO-ACTIVITIES:

In the preliminary screening of protease, amylase and cellulase activity, both the isolates recovered from *Ocimum basillicum* were found to be positive for proteolytic, amylolytic and cellulolytic activity. Further, the isolates recovered from *Syzygium aromaticum* were potent producers of amylase and cellulase. Out of 14 isolates, 7 fungal endophytes were found to exhibit proteolytic activity with maximum potential in #9SASTD

Table 1. Showing culture code of fungal endophyte, plant name, plant part and place of plant collection

S. No.	Culture Code	Plant part	Plant Name	Place of collection
1.	#16OPSTD	Stem	*Ocimum basillicum*	Kanhaiyavihar, Dehradun
2.	#9OPSTITD	Stem internal tissue	*Ocimum basillicum*	Kanhaiyavihar, Dehradun
3.	#6WSLD	Leaf	*Withania somnifera*	Kanhaiyavihar, Dehradun
4.	#9 WSLD	Leaf	*Withania somnifera*	Kanhaiyavihar, Dehradun
5.	#10 WSLD	Leaf	*Withania somnifera*	Kanhaiyavihar, Dehradun
6.	#14 WSLD	Leaf	*Withania somnifera*	Kanhaiyavihar, Dehradun
7.	#22 WSLD	Leaf	*Withania somnifera*	Kanhaiyavihar, Dehradun
8.	#24 WSLD	Leaf	*Withania somnifera*	Kanhaiyavihar, Dehradun
9.	#7SALD	Leaf	*Syzygium aromaticum*	Kanhaiyavihar, Dehradun
10.	#16SALD	Leaf	*Syzygium aromaticum*	Kanhaiyavihar, Dehradun
11.	#2SASTD	Stem	*Syzygium aromaticum*	Kanhaiyavihar, Dehradun
12.	#9SASTD	Stem	*Syzygium aromaticum*	Kanhaiyavihar, Dehradun
13.	#15SASTD	Stem	*Syzygium aromaticum*	Kanhaiyavihar, Dehradun
14	#16SASTITD	Stem Internal tissue	*Syzygium aromaticum*	Kanhaiyavihar, Dehradun

FIGURE 1. Fungal endophytes isolated from different parts of medicinal plants collected from Uttarakhand

Table 2. Summary of endophytic fungi isolated from different tissues of host plant					
S. No	Host plant	Endophytic fungi			Total
		Leaf	Stem	Stem internal Tissue	
1.	*Withania sominifera*	6	0	0	06
2.	*Syzygium aromaticum*	2	3	1	06
3.	*Ocimum basillicum*	0	1	1	2
	Total	8	4	2	14

Table 3. showing bioactivity profiling of culture filtrates of fungal endophytes expressed as zone size in mm					
S. No	Culture code	Mean zone size (mm)			
		Protease	Cellulase	Amylase	Asparaginase
1.	#16OPSTD	8 ± 0	11 ± 0	7 ± 0	-
2.	#9OPSTITD	11 ± 0	10 ± 0	9 ± 0	-
3.	#6WSLD	-	15 ± 0	12 ± 0	14.33 ± 0.57
4.	#9 WSLD	-	-	-	-
5.	#10WSLD	-	-	-	-
6.	#14WSLD	9 ± 0	16 ± 0	11 ± 0	18.67 ± 0.57
7.	#22WSLD	-	-	9.5 ± 0.70	21.67 ± 0.57
8.	#24 WSLD	-	-	-	-
9.	#7SALD	14.5 ± 0.70	8 ± 0	14 ± 0	-
10.	#16SALD	-	10 ± 0	8 ± 0	16 ± 0
11.	#2SASTD	15 ± 0	10 ± 0	16.5 ± 0.70	11.33 ± 0.57
12.	#9SASTD	15.5 ± 0.57	11 ± 0	13 ± 0	18.33 ± 0.57
13.	#15SASTD	-	10 ± 0	9.5 ± 0.70	-
14.	#16SASTITD	7 ± 0	9 ± 0	11 ± 0	-

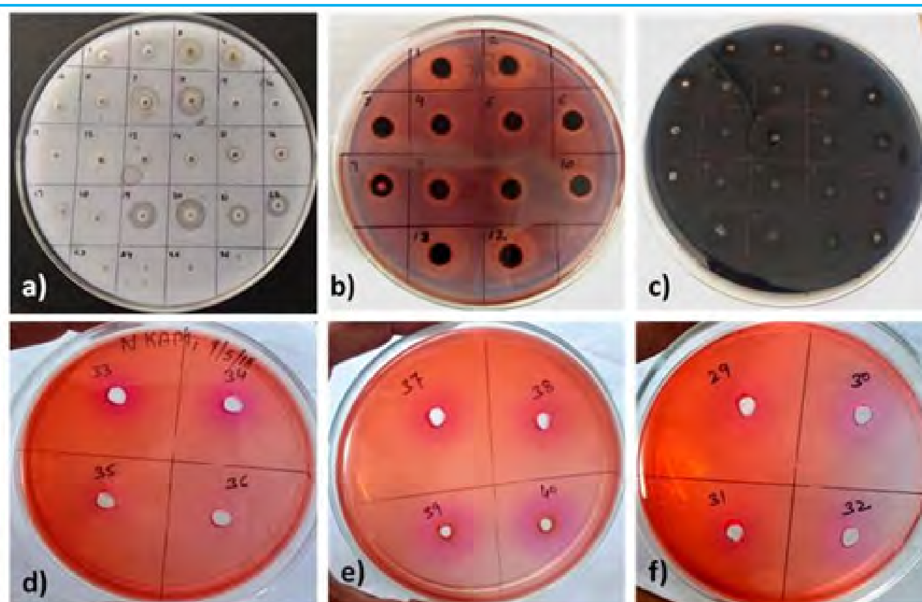

FIGURE 2. Enzyme production by endophytic fungal isolates: a) Proteolytic activity, b) cellulase activity, c) amylase activity, d)-f) L-asparaginase activity of culture filtrates of different fungal endophytes

closely followed by #2SASTD and #7SALD. Furthermore, #14WSLD and #16OBSTD was weak producer of proteolytic enzymes.Further, all the isolates of *Syzygium aromaticum* were found to positive In case of amylase and cellulase activity. However, maximum amylase production was observed in #2SASTD and maximum cellulase production was exhibited by #14WSLF followed by #6WSLF (Table 3, Figure 2). In case of asparaginase production assay, #22WSLD exhibited maximum asparaginase production with zone size of 21.3 mm followed by #14WSLD and #9SASTD with zone size of 18.6 mm and 17.6 mm respectively. Moderate level of enzyme production was observed in #16SALD and #6WSLD with zone size of 16 mm & 14.3 mm respectively (Table 3, Figure 2). Further, the isolates of *Ocimum basillicum* did not showed the activity.

DISCUSSION

Endophytes have been targeted as bioactive repository with immense potential for industrial and pharmaceutical interventions. Among the different types, fungal endophytes have found a niche of being a sublime resource for harnessing novel bioactive agents against spectrum of disorders (Strobel, 2003; Gunatilaka, 2006). Fungal endophytes produce diverse chemistry of molecules depending upon the host plant requirements against different kinds of stresses. Thus, choosing a plant for sampling to isolate a fungal endophyte is a crucial aspect. For this reason the medicinal plants documented in traditional indigenous preparations provide an ample group as repository of fungal endophytes. Among the geographically diverse medicinal plants, high altitude inhabiting medicinal plants have found their own niche in ethnopharmacology (Rajagopal *et al.* 2012). Owing to their immense bioactive potential, high altitude medicinal plants have been exploited to an extent of endangerment. Thus we hypothesis that, high altitude medicinal plants may be explored for endophytic fungal diversity and can further be screened for bioactivities (Chutulo and Chalannavar, 2018)

The present study was undertaken to bio-prospect the fungal endophytes from medicinal of high altitude regions of Uttarakhand for pharmaceutical interventions. *Withania sominifera*, *Ocimum basillicum* and *Syzygium aromaticum* were selected for endophyte isolation based on their ethno-pharmacological potential. We observed higher colonization fungal endophytes in *Withania sominifera* and *Ocimum basillicum*. Isolates were further screened for industrially imperative enzymes. Interestingly, endophytic fungal isolates exhibited varying degree of enzyme production. These results corroborate our hypothesis that high altitude medicinal plants may be explored for isolation of fungal endophytes with potent bioactivities. The present study shows promising signs for further purification and evenness of enzymes which may found application in pharmaceutical industry.

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Antifungal peptides: Biosynthesis, production and applications

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ABSTRACT

Fungal infections in animal, plants and fungal contamination of food for humans and livestock result in substantial worldwide economic losses. In the last few years, fungal infection has increased strikingly by a rise in the number of deaths of acquired immunodeficiency syndrome (AIDS) cancer patients, transplant patients owing to fungal infections. The growth rate of fungi is very slow as compared to bacteria and very difficult to identify. Approximately 100 peptides have been investigated to date for their antifungal properties, which can be of great importance to overcome the human diseases. Insects secrete such compounds, which can be peptides, as a part of their immune defense reactions. Antifungal peptides are excellent models for drug discovery exhibiting unique characteristics such as high specificity, broad spectrum, low level of resistance reaching and unique mode of action. The aim of this review is to provide information on research on these important peptides.

KEY WORDS: ANTIFUNGAL; PEPTIDES; MODE OF ACTION; FUNGAL INFECTION; FUNGI CIDAL

INTRODUCTION

Many research advances have been made in medicine at present. Be it in the treatment of HIV-AIDS, cancer, or organ transplantation, the success rates have increased drastically over past 50 years. Even though success rates have been increased, many patients are left with compromised immune systems (Wisplinghoff *et al.*, 2004). The Patients, receiving chemotherapy, organ transplantation, use of prosthetic Devices and vascular catheters, dialysis etc., are easily susceptible to manybacterial, viral and fungal infections (Spellberg *et al.*, 2008). Even though fungal species are serious pathogens, they get lesser attention when compared to bacterial and viral infec-

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tions as, the frequency of occurrence of fungal infections has been comparatively less to bacterial and viral infections (Georgopapadakou *et al.*, 1996; Wisplinghoff *et al.*, 2004; Porto *et al.*, 2012). Human fungal infections, caused by *Aspergillus fumigatus*, *Cryptococcusneoformans*, *Candida albicans*, are increasing in a number ofimmune-compromised patients (Blanco *et al.*, 2008). Fungal pathogens such as *Candida* species and *Aspergillus* species are more common and account up to 19% of cases (Schelenz *et al.*, 2009). *C. albicans* is known as major fungal pathogen and is 4th most common cause of nosocomial infections (Banerjee *et al.*, 1991; Beck-Sague *et al.*, 1993; Wisplinghoff *et al.*, 2004; Xiao *et al.*, 2013; Chen *et al.*, 2016; Ageitos *et al.*, 2017; Bondaryk *et al.*, 2017).

Only a limited number of antifungal drugs are available such as echinocandins, polyenes etc., (Gupte *et al.*, 2002). Amphotericin B, which was discovered in 1956, is still used for treatment many fungal infections. Just like bacterial resistance, fungal pathogens have also developed resistance in past 20 years. (Gold *et al.*, 2002; Georgopapadakou *et al.*, 1996). The fact that fungal and bacterial infections are different and bacterial infections are treated more easily is because, fungal cells are eukaryotic and bacterial cells are prokaryotic. The main concern in treating fungal infections is that any chemical substance that is successful in damaging the eukaryotic cell wall of fungi may also cause possible damage to human cells, unlike antibiotics, which won't have any effect on humans. Any chemical substance that is toxic to fungus may also be toxic to humans (Mohammad *et al.*, 2015). Therefore, there is need to discover new biochemical targets in fungi. Antifungal peptides are treatment alternatives, derived from natural sources and are effective against fungal infections, thus, safe for immune compromised patients (Gold *et al.*, 2002; Ravi *et al.*, 2011; Thakur *et al.*, 2012; Jia *et al.*, 2016; Wang *et al.*, 2016; Veltri *et al.*, 2017).

Antifungal peptides from natural sources are much cheaper than commercial antifungal drugs and are also better alternative to combat resistance. Antifungal peptides are cationic biomolecules with weight around 1.3 kDa to 30 kDa (Mohammad *et al.*, 2015). Antifungal peptides are classified into two types based on their mode of action. First group are, lytic peptides, (Rees *et al.*, 1997; Shai *et al.*, 1995). These peptides are amphipathic in nature (contain a positive and a neutral charge) and disrupt the membrane structure by fixing onto its surface (Leuschner *et al.*, 2004; Shai *et al.*, 1995). The second group of peptides act by inhibiting the synthesis of cell wall or essential cell wall components such as glucan, chitin (Fernández *et al.*, 2004; Lata *et al.*, 2010; Joseph *et al.*, 2012; Liu *et al.*, 2016; Bondaryk *et al.*, 2017).

SOURCES OF ANTIFUNGAL PEPTIDES

Bacterial Peptides Iturins

Iturin was one of first antifungal peptides, ever isolated. It is produced by different strains of *Bacillus subtilis* (Georgopapadakou *et al.*, 1996). They are cyclic lipopeptides and act by disrupting the cell membrane of fungi, hence leaking its vital ions (XinZhao *et al.*, 2013; Lemaitre *et al.*, 1997). Iturin A, of iturin family, was observed to inhibit *A. flavus* and *F. moniliforme* growth and had Minimal inhibitory concentration (MIC) of 22.0 µg/ml against *Saccharomyces cerevisiae*. It was found to be effective against dermatomycoses. (De Lucca *et al.*, 1999). But iturin A was also observed to be hemolytic. Bacillomycin F, another family member of iturin, is known to inhibit strains such as *Byssochlamys fulva*, *A.niger*, *C.albicans*, and *F.oxysporum*and had MIC of 40.0µg/ml for *A.niger* (De Lucca *et al.*, 1999). Bacillomycin D produced by *Bacillus amyloliquefaciens* was found to be effective against a plant pathogenic fungi *Fusarium graminearum and Candida* species. MIC of (12.5-25) µg/ml was observed against various *Candida* species (Tabbene *et al.*, 2015; Qin Gu *et al.*, 2017).

Syringomycins: Syringomycins are produced by *Pseudomonas syringae* are small cyclic lipodepsipeptides with ergosterol as a binding site in yeast. The most prevalent of Syringomycinsis syringomycin-E (SE) which was found to be lethal to many strains such as *A. flavus, A. fumigatus, A.niger, F. moniliforme and F. oxysporum* showing LD95 of 1.9 µg/ml. it showed MIC of (0.8–12.5) µg/ml against *C. neoformans* (De Lucca et al., 1999). Syringotoxin B, syringostantin A which were lipodepsinonapeptides were found to be effective against *Candida, Cryptococcus*, and *Aspergillus* species. Syringostantin A had MIC of 5.0µg/ml against *A. fumigatus*. Syringotoxin B had MIC of 3.2µg/ml against *C. albicans* (Sorensen et al., 1996; Zhao et al., 2013; Chereddy et al., 2014; Deslouches et al., 2015; Gao et al., 2016; Kubicek-Sutherland et al., 2017).

Pseudomycins: Pseudomycins, another family, structurally related to syringomycins also have antifungal activity against wide ranges of species. Existing as pseudomycins (A, B, and C), these have shown antifungal activity against *Ceratocystis ulmi, C. Albicans, Rynchosporium secalis,Rhizoctonia solani,Sclerotiniasclerotiorum Verticillium albo-atrum, Verticillium dahliae, Thielaviopis basicola, F. oxysporum, F. culmorum. The MIC of pseudomycin A, against* C. *neoformans* was 1.56 µg/ml whereas 3.12 µg/ml was observed against *C. albicans* (De Lucca *et al.*, 1999).

Plant Peptides: Large number of antifungal peptides are identified from plant sources, but only few were tested and found to be effective.

Table 1. Antifungal peptides from bacterial sources

Peptide name	Family/group	Structure	source	Fungal species effected	Typical target organism	Mode of action	In vitro MIC (µg/ml)	Reference
Bacillomycin F	Iturins	lipopeptide	*B. subtilis.*	*Byssochlamys fulva, A. niger, C.albicans, and F.oxysporum*	*A. niger*	lysis	40	(De Lucca et al., 1999; Bionda et al., 2016)
iturin A	Iturins	lipopeptide	*Bacillus amyloliquefaciens*	*A. flavus, F. moniliforme, S. cerevisiae*	*S. cerevisiae*	lysis	22.0	(Georgopapadakou et al., 1996; De Lucca et al., 1999; Brandenburg et al., 2015)
bacillomycin D	Iturins	lipopeptide	*Bacillus amyloliquefaciens*	*F. graminearum and Candida species.*	*Candida species*	*lysis*	12.50-25.0	(Tabbene et al., 2015; Qin Gu et al., 2017,)
syringomycin-E (SE)	Syringomycins	lipodepsipeptide	*Pseudomonas syringae*	*A. flavus, A. fumigatus, A.niger, F. moniliforme and F. oxysporum*	*C. neoformans*	lysis	0.8–12.5	(De Lucca et al., 1999; Falciani et al., 2014)
syringostantin A	Syringomycins	lipodepsinonapeptides	*Pseudomonas syringae*	*Candida, Cryptococcus, and Aspergillus species*	*A. fumigatus*	lysis	5.0	(Sorensen et al., 1996; Falciani et al., 2014)
Syringotoxin B	Syringomycins	Lipodepsinonapeptide	*Pseudomonas syringae*	*Candida, Cryptococcus, and Aspergillus species.*	*C. albicans*	lysis	3.2	(Sorensen et al., 1996; Lyu et al., 2016)
pseudomycin A	Pseudomycins	lipodepsinonapeptides	*Pseudomonas syringae*	*C. albicans, F. oxysporum , F. culmorum, C. neoformans*	*C. albicans*	lysis	3.12	(De Lucca et al., 1999; Brunetti et al., 2016)

Table 2. Antifungal peptides from plant sources

Peptide name	Family/group	No. of amino acids	source	Target organism	In vitro MIC (µg/ml)	Reference
Ib-AMP3	Plant defensins	20	*Impatiens balsamina*	*F. moniliforme*	50.0	(De Lucca et al., 1999; Asano et al., 2013)
Frangufoline	Cyclopeptides	*534	*Rhamnus frangula*	*A. niger*	5.0	(Gournelis et al., 1997; De Lucca 2000; Tan et al., 2006; Choe et al., 2015)
Rugosanine A	Cyclopeptides	*585	*Ziziphus rugosa*	*A. niger*	5.0	(Gournelis et al., 1997; De Lucca 2000; Tan et al., 2006; Cole et al., 2016)
Nummularine	Cyclopeptides	*587	*Ziziphus nummularia*	*A. niger*	5.0	(Gournelis et al., 1997; De Lucca 2000; Tan et al., 2006; Dobson et al., 2014)
ACE-AMP1	Lipid transfer proteins	93	*Allium cepa L*	*F. oxysporum*	10.0	(De Lucca 2000; Dutta et al., 2015)

Plant defensins

Plant defensins are eight disulfide-linked cysteines with a single helix and triple-stranded b-sheet (Bruix *et al.*, 1995). Ib-AMP_3, isolated from *Impatiens balsamina,* was observed to be lethal against germinated conidia of *A. flavus* by 42%, where as it was non-lethal against non-germinated conidia.It had MIC of 50.0µg/ml against *F. moniliforme* (De Lucca *et al.*, 1999; Asano *et al.*, 2013).

Table 3. Antifungal peptides from fungal sources

Peptide name	Structure	source	Typical target organism	Mode of action	In vitro MIC (µg/ml)	Reference
Caspofungin	lipopeptide	*G.lozoyensis*	*Candida spp*	*glucan synthesis*	8 - 64	(Bartizalet al., 1997;Groll et al., 1999; Kuhn et al., 2002; *Deresinski* et al., *2003*; Porto et al., 2012)
Anidulafungin (LY303366)	Lipopeptide	*A. nidulans*	*Candida spp*	*glucan synthesis*	0.5 - 4.0	*(Lucca et al., 1999; Denning et al., 1997;* Ghannoum et al., 2005; De *Lei* et al., *2013)*
Cilofungin (LY121019)	Lipopeptide	*A. nidulans*	*C. albicans*	*Glucan synthesis*	0.62	(De Lucca 2000; Joseph et al., 2012)
Echinocandin B	Lipopeptide	*A. nidulans*	*C. albicans*	*Glucan synthesis*	0.625	(De Lucca 2000; Veltri et al., 2017)
Aculeacin	Lipopeptide	*A. aculeatus*	*C. albicans*	*Glucan synthesis*	0.2	(De Lucca et al., 1999; Chen et al., 2016)
Trichopolyn	Amino-lipopeptide	*Trichoderma polysporum*	*C. albicans*	*Unknown*	0.8	(De Lucca 2000; Liu et al., 2016)
Leucinostatin	Amino-lipopeptide	*Penicillium lilacinum*	*C. neoformans*	*Unknown*	0.5	(De Lucca 2000; Zhao et al., 2013)

Table 4. Antifungal peptides from insect sources

Peptide name	Family/group	No. of amino acids	source	Typical Target organism	Mode of action	In vitro MIC (µg/ml)	Reference
Cecropin A	Cecropins	37	*Hyalopora cecropia*	*F. oxysporum,*	lysis	12.4	(De Lucca et al.,1998; Joseph et al., 2012)
Cecropin B	Cecropins	35	*Hyalopora cecropia*	*A. fumigatus*	lysis	9.5	(Nappi et al., 2001; Xiao et al., 2013)
Drosomycin	Cysteine-rich peptides	44	*Drosophila melanogaster and Podisus maculiveris*	*F.oxysporum*	lysis	5.9	(De Lucca, 2000; Veltri et al., 2017)
Thanatin	Cysteine-rich peptides	21	*Podisus maculiveris*	*F. oxysporum*	Unknown	5.0	(Bulet et al., 2005; Wang et al., 2015)
Heliomicins	Insect Defensins	44	*Heliothis virescens*	*C. neoformans*	Unknown	12.0	Nappi et al., 2001; De Lucca 2000; Zhao et al., 2013; Ageitos et al., 2017)

Cyclopeptides: Cyclopeptides from different species of *Rhamnaceae* family were observed to have antifungal activities. Frangufoline, from barks of *Rhamnus frangula* were observed to have anti-bacterial and anti-fungal properties. It showed MIC of 5.0 µg/ml for *A. niger*. Nummularine (B, K, R, and S), from stem barks of *Ziziphus nummularia*, Rugosanine (A and B) from stem barks of *Ziziphus rugosa* and abyssenine-C from stem barks of Ziziphus abyssinica, were all observed to have antifungal properties against *A. niger* with MIC of 5 µg/ml. However, they were observed to be well effective against *A. niger* but not against *C. albicans* and their mechanism of action was also unknown (Gournelis *et al.*, 1997; De Lucca 2000; Tan *et al.*, 2006).

Lipid transfer proteins and other peptides: ACE-AMP1 is a lipid transfer protein, produced by seeds of *Allium cepa* which was observed to be effective against *F. oxysporum* with MIC of 10.0 µg/ml (Cammue *et al.*, 1995; De Lucca 2000). Apart from the above antifungal peptides, some other peptides include, Chitinases and glucanases, which hydrolyze chitin, glucan, and

Table 5. Antifungal peptides from amphibian sources

Peptide name	No. of amino acids	source	Typical Target organism	Mode of action	In vitro MIC (µg/ml)	Reference
Magainin 2	23	*Xenopus laevis*	*C. albicans*	Lysis	80.0	(Zasloff et al., 2002; Bondaryk et al., 2017)
Dermaseptin b	27	*Phyllomedusa sauvagii*	*C. neoformans*	Lysis	60.0	(Landon et al., 1997; Brandenburg et al., 2015)
Dermaseptin s	34	*P. sauvagii*	*C. neoformans*	Lysis	5.0	(Landon et al., 1997; Brunetti et al., 2016)
Skin-PYY (SPYY)	36	*P. bicolor*	*A. fumigatus*	Membrane permiation	80.0	(Vouldoukis et al., 1996; Brunetti et al., 2016)
Brevinin-2R	24	*Rana ridibunda*	*C. albicans*	–	3.0	(Conlon et al., 2003; Anunthawan et al., 2015)

the essential cell wall components of fungi. Prematins, members of PR-5 protein family, act by permeabilizing fungal membranes. Similarly, Thionins inhibit by permeabilizing fungal membranes and were found to be effective against *F. graminearum and F. sporotrichioides* (Velazhahan *et al.*, 2001; Asano *et al.*, 2013).

Fungal Peptides: Antifungal peptides from fungi are more active than those compared to bacteria and plants. Echinocandins are lipopeptides which inhibit 1,3-β-glucan synthase (Gregory et al., 2007). Glucan is the major component of cell wall of fungi and inhibition of glucan may result in osmotic instability and in cell lysis. (Lee et al., 1995; Gregory et al., 2007; Osorio et al., 2015; Liu et al., 2016). The MIC90 value of echinocandins was found to be ≤2 μg/mL against *Candida* spp (Zaas et al., 2005). A-192411.29 had anti- fungicidal activity against *C. albicans, C. tropicalis* and *C. glabrata* (Vazquez et al., 2005; Kaconis et al., 2011; Chu et al., 2013). But, the echinocandins do not show any antifungal activity against *Cryptococcus* spp, *Trichosporon* spp, *Fusarium spp*, zygomycetes (Zaas et al., 2005; Kazemzadeh-Narbat et al., 2010). They also, do not affect human cells, as human cells do not contain 1,3-β-D-glucan. However, echinocandins are labeled category C and are toxic to embryos (Gregory et al., 2007; Lakshmaiah Narayana et al., 2014).

Micafungin from *Coleophoma empedra*, caspofungin from *Glarea lozoyensis* and anidulafungin from *A. nidulans* of echinocandin family have been approved so far (Murdoch et al., 2004; Montgomery et al., 2013). Of these, anidulafungin displays least MIC values followed by micafungin and caspofungin being most. This was observed against *Candida* spp. (Zaas et al., 2005; Mojsoska et al., 2015). Caspofungin, also known as (MK-0991) is a second generation pneumocandin from Glarea lozoyensis (Abruzzo et al., 1997; Groll et al., 1999; López-Garcia et al. 2005; Popovic et al., 2012). It was fungicidal against *C.albicans* and *C. parapsilosis* (Bartizal et al., 1997; Kuhn et al., 2002; Deresinski et al., 2003; Ordonez et al., 2014). It was observed be effective against hyphal tips *A. fumigatus* although not completely lethal (Krishnan et al., 2005). It was also lethal against several molds such as *Alternaria* sp., *Curvularia* sp., *Acremonium* sp., *Bipolaris* sp., and Trichodermasp (Kahn et al., 2006). Micafungin also known as FK463 had antifungal activity against disseminated candidiasis and aspergillosis (Petraitiset al., 2000; Lakshmaiah Narayana et al., 2015).

The optimal concentration of FR463 at single infusion was observed to be 2.5-25 mg (Azuma et al., 1998; Pettengell et al., 1999; Kasetty et al., 2015; Kang et al., 2017). Anidulafungin (V-echinocandin), previously known as LY303366 is a semisynthetic echinocandin currently used as antifungal drug (*Krause* et al., *2004;* Harder et al., 2013; Kang et al., 2017).It is a lipopeptide produced by A. nidulans, (*Lei* et al., *2013*) and acts by inhibiting glucan synthase (*Denning* et al., *1997;* Anunthawan et al., 2015). It was observed to be effective against Candidemia and other Candida infections and esophageal candidiasis. MIC of (0.5 to 4.0) μg/ml was observed in *Candida* spp. However, Anidulafungin displays low MICs against strains of *C. parapsilosis* and is not effective inactive against *C. neoformans* and Blastomyces dermatitidis *(*De Lucca et al., 1999; Ghannoum et al., 2005; Ben Lagha et al., 2017*)*.

Echinocandin B from *A. nidulans* and *A. rugulosus* was effective against *C. albicans* with MIC of 0.625 μg/ml. Cilofungin (LY121019), isolated from Aspergillus spp. had MIC of 0.62 μg/ml. Amino-lipopeptides such as Trichopolyns from Trichoderma polysporum have MIC of (0.78 - 6.25) μg/ml for *C. albicans*. Other families of potent antifungal peptides include the leucinostatins and helioferins families also consist of antifungal poperties, but, where toxic, hemolytic to mammalian cells in vitro (De Lucca 2000; *Lei* et al., *2013;* Osorio et al., 2015; Chen et al., 2016; Ageitos et al., 2017).

Insect Peptides: Cecropins

Cecropins (A and B) are linear lytic peptides, made up of an 11- amino acid sequence, produced in hemolymph giant silk moth, *Hyalopora cecropia*. Cecropin B was observed lethal against F. oxysporum (approximately 95%), A. fumigatus 9.5 μg/ml (De Lucca *et al.*, 1998; Nappi *et al.*, 2001). cecropin A was observed to be more fungicidal at neutral pH and was more affective against Fusarium moniliforme and Fusarium oxysporum with total killing of 12.4 μg/ml (De Lucca *et al.*,1998).

Drosomycin: Drosomycin is a Cysteine-rich peptide containing 44 amino acid with a twisted three-stranded sheet structure steadied by disulfide bonds. It is isolated from Drosophila melanogaster and *Podisus maculiveris* and was found to be effective against F.oxysporum with MIC value of 5.9 μg/ml (De Lucca, 2000).

Glycin-rich peptides

Antifungal peptides, such as holotricin-3, and tenecin-3 are glycine-rich peptides isolated from insects (Nappi *et al.*, 2001). Tenecin-3 was studied to be effective against *C. albicans* (Ganz, 2003). Holotricin-3, was isolated from larval hemolymph of Holotrichia diomphalia, and was observed to inhibit *C. albicans* growth (Lee *et al.*, 1995).

Thanatin: Thanatin is another non-hemolytic Cysteine-rich peptide containing 21 amino acid residues and is smaller compared to drosomycin. It was affective against many strains such as *Trichoderma viride, Alternaria brassicola, Neurospora crassa, Botrytis cinerea, and Fusarium culmorum, A. fumigatusT.* mentagro-

phytes and F. *oxysporum* (Fehlbaum *et al.*, 1996; Bulet *et al.*, 2005). MIC of 5.0 µg/ml was observed against *F. oxysporum*. However Thanatin was not effective against yeast (Mandard *et al.*, 1998).

Heliomicin: Heliomicin from *Heliothis virescens* (tobacco budworm), was observed to have antifungal activity against *C. neoformans*, with MIC of 12.0 µg/ml (De Lucca 2000; Nappi *et al.*, 2001).

Amphibian Peptides: Magainins: Magainins was the first among the antifungal peptides from amphibian sources. They are amphiphilic, non-hemolytic and are produced by *Xenopus laevis* (African clawed frog). Magainin 2 inhibited *C. albicans* growth and had MIC of 80.0 µg/ml (De Lucca *et al.*, 1999; Zasloff *et al.*, 2002).

Dermaseptins: Dermaseptins are linear, lytic,peptides produced by *Phyllomedusa sauvagii* (South American arboreal frog). Dermaseptin was lethal towards for *A. flavus, A. fumigatus*, and *F. oxysporum*, with LD50 values observed as 3 µM, 0.5 µM, and 0.8 µM, respectively (Landon *et al.*, 1997). Dermaseptin b was effective against yeasts and some filamentous fungi such as *C. neoformans*and had MIC value of 60.0µg/ml. Dermaseptin s had MIC of 5.0µg/mlfor *C. neoformans*. (De Lucca *et al.*, 1999).

Skin-PYY (SPYY): Skin-PYY (SPYY), is an antifungal compound produced by *Phyllomedusa bicolor* (South American tree frog). It was observed to inhibit *C. neoformans*, *C. albicans*, and *A. fumigatus* and had MIC values of 20 µg/ml, 15 µg/ml, and 80 µg/ml, respectively (Vouldoukis *et al.*, 1996).

Brevinin: Brevinin-2R isolated from skin of Rana ridibunda (red frog). It is non-hemolytic, 24 amino acid peptide with α-helical conformation. It was observed to have MIC of 3.0 µg/ml against *C. albicans* (Conlon *et al.*, 2003).

FUTURE PROSPECTS

Emerging fungal resistance to conventional therapies necessitates the development of novel antifungal strategies. In this context, Anti-fungal peptides draw the attention as alternative potential antifungal agents (Brunetti et al., 2016). These peptides are relatively safe, tolerated and highly effective. As per the information available in the literatures, only few antifungal peptides are used in antifungal therapy (Brandenburg et al., 2015). There are various problems addressed which is limiting the uses of these peptides, such as low bioavailability, hemolytic activity, instability, high cost of production, possible aggregation, loss of activity in high salt concentrations, poor ability to cross physiological barriers (Chen et al., 2016; Ageitos et al., 2017).

Due to these effects, the therapeutic use of antifungal peptides is significantly decreased now a day. However, the utilization of these peptides could be enhanced by chemical optimization and new delivery strategies. With the advancement of new research strategies, the wide variety of natural antimicrobial peptides should be characterized both structurally and functionally for making them extremely promising source of ideas in design the novel antifungal peptides. In particular, application of dendrimers as scaffolds for assembling well defined macromolecular polyvalent molecules or synthesis de novo of per se active linear and branched peptide mimics makes them extremely promising for use as new generation antifungal peptides.As found in several studies, the modes of antifungal action must be well understood (Deslouches et al., 2015; Gao et al., 2016; Kubicek-Sutherland et al., 2017). Hopefully, all these efforts will result in the development of a novel class of antifungal agents to their full potential.

CONCLUSION

Antifungal peptides are excellent models for drug discovery exhibiting unique characteristics such as low level of resistance reaching the absent, high specificity, broad spectrum, and unique mode of action. Despite the distinctiveness, only few examples of antifungal peptides have successfully reached the market.

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Comparative studies on the physicochemical and microbiological characteristics of different animal milk collected from the farms of Khartoum State, Sudan

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ABSTRACT

Milk is considered as one of the finest natural foods containing all the essential nutrients such as lactose, fat, protein, minerals and vitamins in balanced ratio rather than the other foods. The present study was aimed to compare the physicochemical and microbiological qualities of cow, camel and goat milk farmed in Khartoum states. Milk samples were collected from the villages of Khartoum, Sudan and analyzed for nutritional and mineral variations. Our proximate analysis showed that, there was no significant difference ($p > 0.0\ 5$) in total solid content, moisture and fat content of cow, camel and goat milk. However, we found significant differences ($p > 0.0\ 5$) in cow, camel and goat milk for protein content, ash content, total acidity content and pH. In addition to that, mineral analysis of entire samples revealed that level of potassium was highest followed by calcium and phosphorus. However, level of magnesium and zinc was found to be lowest in concentration. Microbiological investigation showed that, total viable count in cow, camel and goat was 3.5×103, 7.5×103 and 4.5×103, respectively. Moreover, total coliform count was found to be 9, 7 and 6 in cow, camel and goat respectively count indicates possible poor hygienic practices at farm level. In addition to that, Yeast and Mold, *Staphylococcus aureus, Escherichia coli* and *Salmonella* was not detected in the samples. This work provides updated information on the current physicochemical and microbiological characteristics of cow, camel and goat milk collected from the farms of Khartoum, Sudan. This can be of great importance in the fields of dairy, food technology and food analysis.

KEY WORDS: MILK, PHYSICOCHEMCIAL ANALYSIS, MINERAL ANALYSIS, SUDAN

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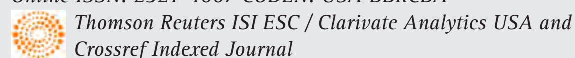
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INTRODUCTION

Milk is one of the utmost important nutritious foods consumed throughout the world. It has been considered as key sources of all basic nutrients required for mammals including human beings. Milk is a complex colloidal dispersion containing fat globules, casein micelles and proteins in an aqueous solution of lactose, minerals and a few other minor compounds. Moreover, major chemical composition of milk has been reported in the form of water, carbohydrate, fat, protein, organic acids, enzymes and vitamins. Though, its physical and chemical properties depend on intrinsic compositional and structural factors, extrinsic factors such as temperature, diet and type of breed as well as post-milking treatments. In addition to that, milk from several animals such as cow, camel and goat etc. are used for various nutritional purposes such as feeding to young ones or used for dairy preparation such as milk cream, butter, yogurt, ghee, sour milk, etc., (Imran *et al.*, 2008, Sood *et al.*, 2016).

The health benefits of milk and other dairy products are known to humanity since ancient times and could be attributed due to naturally occurring active compounds that are existing in milk. Milk facilitates postnatal adaptation of baby through digestive maturation simultaneously by providing the bioactive components and nutrients. It supports lymphoid tissues development and in the establishment of symbiotic micro flora. The importance, potency and the quantity of milk bioactive compounds are possibly more than old consideration. They comprise certain specific organic acids, vitamin A, B12, D, riboflavin calcium, carbohydrates, phosphorous, selenium, magnesium, zinc, proteins, bioactive peptides and oligosaccharides (Ahmad *et al.*, 2011; Homayouni *et al.*, 2012, Gasmalla *et al.*, 2017).

According to the Ministry of Livestock of Sudan (North African Country), an estimated 4.8 million ton of milk per year is produced of which 50% is used for direct human consumption and the remaining for bakeries and for feeding young stock. The average consumption is 6.5 million liters per day, which equals 0.18 liter (one cup of milk)/capita per day or 66 liter per year. Most of this milk is consumed directly without processing. Moreover, previous study shows major concerns for cow milk only, which represent 84 % of the milk consumed around the world and to a lesser extent to other animal likewise camel and goat milk despite of their high nutritional importance (Dairy Quick Scan Sudan, 2016, Mayilathal *et al.*, 2017).

Camel milk has been considered as an important source of proteins for the people living in the arid lands of the world. Its medicinal properties, has been widely exploited for human health. Camel milk is considered to possess anti-cancer, hypo-allergic and anti-diabetic properties. A high content in unsaturated fatty acids contributes to its overall dietary quality (Konuspayeva *et al.*, 2008). On the other hand, goat is considered as the poor man's cow due to its great contribution to the health and nutrition of the landless and rural people. Consumption of goat milk should be enhanced because of its therapeutic properties and nutrition value (Mayilathal *et al.*, 2017).

Goat was one of the first animals to be domesticated and it remained a popular animal serving the needs of human beings all over the world. Goat milk is one of the milk sources that was characterized as an economical, nutritional and medical importance, especially for children who suffer allergy from the cow milk. In addition to that, goat milk has high importance as a source of nutrition for poor communities (Warsama *et al.*, 2015). Goat milk has high biological value and nutritional qualities due to its higher digestibility and its dietary characteristics with smaller diameter fat globules. It presents a chemical composition composed of proteins of high biological value and essential fatty acids, besides its mineral and vitamin content (Nascimento *et al.*, 2017).

Based upon the nutritional facts and importance of animal milk such as cow, camel and goat their quality needs further exploration and updated information. The present study was carried out to evaluate and compare the physicochemical and microbiological properties of cow, camel and goat milk collected from the farms of Khartoum State, Sudan.

MATERIAL AND METHODS

Collection of sample:

A total of 9 fresh milk samples were collected during the month July–September 2017 from the individual animals (cow, camel and goat) by using hand milking and milk samples collected in separate sterilized containers used for sample collection. Then the collected samples were immediately transferred to an ice-box and transported to the laboratory for further chemical and microbiological study.

Proximate Analysis:

Total solid, moisture content, Lactose content and fat was measured as per method mentioned (El-Hag *et al.*, 2013; Ashraf *et al.*, 2016). Total protein was measured according to Kejldahl method using factor 6.38 (Bashir *et al.*, 2015). pH, titratable acidity and ash was measured according to AOAC (2006) methods.

Microbiological Analysis:

Microbiological quality (Total viable count, yeast and mold, Staphylococcus aureus, coliform, Salmonella and E. coli) of the products were investigated by ISO (International Organization for Standardization) methods ISO:

4833-2003, ISO: 21527-(2)2008, ISO: 6888-(1) 1999, ISO: 4832-2006, ISO: 6579-2004 and ISO: 6888-(1) 1999 respectively.

Determination of Milk Minerals : 2 gram milk sample and 10 ml of 20% Nitric acid (HNO_3) were added to crucible. The mixture was initially heated at 70–85°C for 2 hours and later on placed into muffle furnace for another 3 hours. In addition to that, after completion of sample digestion the content of crucible was filtered using Nalgene filter (Thermo scientific) unit. The filtrate was collected in 100 ml volumetric flask and allowed to cool. After cooling the volume was made up to 100 ml using deionized water and analyzed with ICP-MS (Inductive Coupled Plasma-Mass Spectrometry). For the sample preparation all the glassware was washed with deionized water and rinsed three times with 20% nitric acid. Milk minerals were determined for calcium, phosphorous, sodium, potassium, magnesium, zinc, iodine and cobalt (Alghamdi *et al.*, 2018).

Statistical Analysis:

All the experiments were carried out in triplicates. The data were analyzed statistically with SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean was statistically compared by student t test at P <0.05% level.

RESULTS AND DISCUSSION

Variations in the composition of cow, camel and goat milk collected from the farms of Khartoum state, Sudan. Physicochemical investigations were presented in table 1. Moreover, mineral analysis showed a significant variation as depicted in table 2. In addition to that, table 3 represents microbiological analysis.

Table 1. Chemical composition of cow, camel and goat milk

Parameters \ Sample	Cow	Camel	Goat
Moisture content (%)	86.34±0.0[a]	87.28±0.09[a]	87.71±0.07[a]
Fat content (%)	3.63±0.01[b]	4.3±0.01[a]	3.90±0.12[b]
Protein content (%)	3.42±0.10[b]	3.83±0.15[a]	4.81±0.12[a]
Ash content (%)	0.813±0.06[a]	0.653±0.05[b]	0.899±0.34[a]
T.S.S (%)	15.0±0.05[a]	15.28±0.01[a]	14.66±0.057[a]
pH	6.42±0.01[b]	6.32±0.05[c]	6.47±0.05[a]
Acidity (%)	0.230±0.06[c]	0.183±0.08[b]	0.153±0.03[a]
Lactose (%)	3.19±0.01[b]	4.04±0.05[a]	3.79±0.7[c]

Table 2. Mineral composition of cow, camel and goat milk

Parameters \ Sample	Cow	Camel	Goat
Calcium (mg/100 g)	119.90±0.69	119.27±3.43	127±3.42
Phosphorus (mg/100 g)	95.03±0.72	83.58±7.64	66.36±9.91
Magnesium (mg/100 g)	13.42±0.24	12.43±0.94	11.86±1.28
Sodium (mg/100 g)	49.67±0.70	68.58±3.52	41.91±7.02
Potassium (mg/100 g)	147.02±1.55	160.50±9.23	133.57±10.42
Zinc (mg/100 g)	0.38±0.00	0.519±o.o35	0.326±0.030
Cobalt (mg/100 g)	0.0599±0.007	0.0638±0.0069	0.0559±0.0084
Iodine (mg/100 g)	0.0336±0.004	0.0357±0.0067	0.0317±0.0049

Table 3. Microbiological analysis of cow, camel and goat milk

Parameters \ Sample	Cow	Camel	Goat
Total viable count (CFU ml^{-1})	3.5 × 103	7.5 × 103	4.5 × 103
Yeast and Mold (CFU ml^{-1})	ND	ND	ND
Staphylococcus aureus (CFU ml^{-1})	ND	ND	ND
Total Coliforms (CFU ml^{-1})	9	7	6
E. Coli (CFU ml^{-1})	ND	ND	ND
Salmonella 25ml	ND	ND	ND

Total solids and Moisture content: Our results showed that, the total solid content for cows, camels and goats milk in the present study were 15.0±o.05a%, 15.28±o.01a and 14.66±0.o57a%, respectively. The statistical analysis of the data showed non-significant differences (p > 0.0 5) between cows, camels and goats milk. However, moisture analysis showed that, cow, camel and goat milk in the present study were 86.34 ± 0.0a, 87.28 ± 0.09a and 87.71 ±0.07a respectively as shown in table 1. The statistical analysis of the data showed no significant difference (p≤0.05) between three species and it was similar between them. Ealier reports reveal that, the total solid content in these milk was slightly lower than the observed results (Konuspayeva *et al.*, 2008; Elsheikh *et al.*, 2016).

Lactose content: The lactose content of cow, camel and goat milk in the present study were found to be 3.19±0.01%, 4.04±0.05%, 3.79±0.7%, respectively as shown in Table 1. The lactose content of cows, camels and goats milk in the present study revealed that there was significant difference (p≤ 0.05) between cows, camels and goats milk. The lactose of camels milk in the present study was found to be similar to the previous reports (Konuspayeva *et al.*, 2008). Moreover, lactose content in goat milk was not significant with the pervious study ie; 5.0**±**0.0**4**% (Warsama *et al.*, 2015).

Fat: The content of fat in cow, camel and goat milk in the present studies were 3.63**±0.01**%, 4.3**±0.01**% and 3.9**±0.12**% respectively as shown in table 1. The statistical analysis showed, no significant difference (p > 0.05) between cow , camel and goat milk. The milk fat of camels support the findings determined by Shuiep *et al.*, 2014. However, fat content in cow milk was similar to previous study ie; 3.70% (Mourad *et al.*, 2014). Moreover, the fat percentage in different milk samples could be due to genetic and environmental factors.

Protein: The content of the milk protein for cows, camels and goats were found to be 3.42±0.10b, 3.83±0.15a, 4.81±0.12a respectively as shown in Table 1. The statistical analysis of the data showed significant difference (p > 0.05) between cows, camels and goats milk. This results was in accordance with the previous study (Mourad *et al.*, 2014).

pH and Acidity: Table 1 shows that, the results of pH for cows, camels and goats milk in the present study were 6.42±o.01, 6.32±0.05 and 6.47±0.o5, respectively. In addition to that, acidity for cows, camels and goats milk were found to be 0.230±o.06%, 0.183±0.08% and 0.153±0.o3% respectively. The content of the pH and Acidity of cows, camels and goats milk in the present study the data revealed that there was significant difference (p≤ 0.05) between cows, camels and goats milk. Our results were in accordance with the previous study (Fahmid *et al.*2016).

Ash: Our results shows that, cow, camels and goats milk had 0.813±0.0%, 0.653±0.05% and 0.899 ±0.034% of ash respectively as shown in Table 1. The statistical analysis of the data showed significant difference (p≤0.05) between camel from other species. The content of milk ash of camels in present study was similar to the previous study (Mourad *et al.*, 2014).

MINERAL ANALYSIS

The results of calcium (Ca), Phosphorus (P), Magnesium (M), Sodium (Na), Potassium (K), Zinc (Zn), Cobalt (Co) and Iodine (I) for cow, camel and goat milk were presented in table 2. The data indicated that there was highly significant difference (p≤ 0.001) between goats with cow and camel milk. Goat milk had higher amount of calcium level than camel milk. The variation of Ca concentration in milk could be due to species, absorption and availability of Ca to the animal, environmental factors. Moreover, results of phosphorus showed a significant difference at (p ≤ 0.05) between cows and other two species . No significant difference between camels and goats. The level of P in camel milk in present study was higher than values reported (Zamberlin *et al.*, 2012). However, result of magnesium shows that, there was a significant difference (p ≤ 0.05) between Mg level of cows , camels and goats milk. The camel milk exhibited higher Mg level than goat milk. The level of Mg in camel milk in the present study was similar for the value demonstrated by (Konuspayeva, *et al.*, 2009). Similarly, results of sodium and potassium showed highly significant different at (p ≤ 0.01) in Na concentration between cows, camels and goats but no significant difference (p>0.05) in K concentration. The results of potassium, in the current study showed that there was no significant difference among the cows, camel and goats milk. The levels of micro minerals of cow milk in present study were Zn 0.38±0.00, Co 0.0599±0.007 and I 0.0336±0.004, camels milk Zn 0.516±0.035, Co 0.0638±0.0070, and I 0.035±0.0072 mg/100ml. Where, as the levels of micro minerals of goats milk Zn 0.326±0.030, Co 0.0559±0.00837, and I 0.035±0.0072. The data revealed that, there was no significant difference (p > 0.05) between camels and goats milk in Zn, Co and I concentrations.

MICROBIOLOGICAL ANALYSIS

The microbiological analysis results of cow, camel and goat milk were presented in table 3. Our data shows that, *Salmonella spp* were not detected in the milks. However, the number of total viable count in cow, camel and goat milk were found to be $3.5x10^3$, $7.5\ x10^3$, $4.5x10^3$ CFU ml^{-1}, respectively. Moreover, total coliform count was found to be 9, 7 and 6 in cow, camel and goat respectively count indicates possible poor hygienic practices at

farm level. In addition to that, Yeast and Mold, *Staphylococcus aureus*, *E. Coli* and *Salmonella* was not detected in the samples.

CONCLUSION

Milk is an essential product for human consumption as it is a complete food supplement in various parts of the world. Milk can be obtained from different animal species, such as goats, cows and camels. Our study focuses on the nutritional and microbiological characterization of cow, camel and goat milk. We found that, cow, camel and goat milk contained similar percentages of protein implying that any of them can adequately serve as a nutritional source of protein for human consumption. However, lactose and fat percentage had significant variation. In addition to that, the level of potassium was highest among the entire mineral tested. Detection of coliform and total viable count indicates poor hygienic conditions during the milking process by farmers. Furthermore, intensive studies should be carried out to investigate the vitamin and other micro nutrients present in milk. This work provides updated information on the physicochemical and microbiological characteristics of cow, camel and goat milk collected from the farms of Khartoum, Sudan. This can be of great importance in the fields of dairy, food technology and food analysis.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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Isolation, phenotypic and genotypic characterization of indigenous *Beauveria bassiana* isolates from date palm infested with *Rhynchophorus ferrugineus* in Hail region, Saudi Arabia

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ABSTRACT

The red palm weevil (RPW) *Rhynchophorus ferrugineus* overrun date palm ranches in many parts of Saudi Arabia, henceforth causes massive economic losses. Integrated pest Management (IPM) of the RPW by utilizing Entomopathogenic fungi (*Beauveria bassiana*), which has antagonistic activity against many insect pests, was the main objective of the current study. In the present study, soil samples, samples of dead red palm weevils (RPW) and palm fronds were collected according to RPW incidence map of Hail region, Saudi Arabia. Isolates of entomopathogenic fungi were isolated from the dead RPW adults and larvae. The fungal culture (BSA1, BH-2 and BH-3) was preserved and maintained for further analysis. Morphological and biochemical characterization of the antagonist fungi were employed and confirmed that the fungus belonged to *Beauveria* spp. Further, this fungal isolates were propagated and prepared for genetic characterization. Sequencing of internal transcribed spacers (ITS1 and ITS2) region was shown that three polymorphic ITS regions. The molecular identification of the fungus strain was employed at King Faisal University-the College of Agriculture and Food Science and confirmed that the fungus identified as *Beauveria bassiana* as the first record of this beneficial species in Hail region.

KEY WORDS: DATE PALM, RED PALM WEEVIL, FUNGUS, MORPHOLOGICAL, BIOCHEMICAL, MOLECULAR IDENTIFICATION

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INTRODUCTION

The red palm weevil (RPW) *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), is an overwhelming palm pest that can cause tremendous financial losses worldwide throughout the previous 30 years, as they are notable to assault several species (more than 200) of palm including the date palm (*Phoenix dactylifera* L.) (Murphy and Briscoe, 1999; Barranco *et al.*, 2000; Faleiro, 2006). These large economic losses in date palms could ascribed to the way that, to-date, there are no powerful control measures. *Rhynchophorus ferrugineus* is broadly geographically distributed in Africa, Asia, Europe, Oceania, and North America (EPPO, 2006; 2007a; 2007b; 2009; Azmi *et al.*, 2017).

The entomopathogenic fungus *Beauveria bassiana* (*Ascomycota*, *Hypocreales*) has demonstrated use within insect biocontrol administrations for concealment of numerous crop. It infects a an extensive variety of insect pests of socioeconomic importance pests (Bing & Lewis 1991, 1992, Krueger & Roberts 1997; Mulock & Chandler 2000). Recently, *B. bassiana,* was additionally found to occur naturally as an endophyte in plant tissues, for example, as leaves, twigs, wood and bark in specific plants like maize, cotton, wild cacao, white pine, coffee and furthermore been set up as an endophyte artificially in specific crops like, maize, cotton, tomato, opium poppy, cacao, coffee, date palm, banana, sorghum and jute. The entomopathogenic fungus *B. bassiana* isolated from dead *R. ferrugineus* cadavers gave more mortality compared to the other isolates. In the virulence bioassay two isolates of *B. bassiana* shown the highest percentage of larval and adult mortality at all exposure which recommend that they may be the most effective isolates for sustainable insect control programs, (Yasin *et al.*,2017).

Endophytes have several advantages; firstly, they are inside the plant tissues and constantly shielded from abiotic push factors. Also, the application cost is less because of limited application through seed treatment or seedling dip or foliar spray. Moreover, once settled as an endophyte, they may offer season-long protection against the pests that have secretive life cycle by causing encouraging discouragement or antibiosis (Vega *et al.*, 2008) or prompting their mortality or less pervasion. All these properties of fungal endophytes make them reasonable to be utilized as a bio-control agent to protect crops from the pests. Insect pests such as the European corn borer (*Ostrinia nubilalis*) in U.S.A., and the banana weevil (*Cosmopolites sordidus*) in Uganda respectively were effectively controlled by endophytic establishment of *B. bassiana*.

Date palms are considered as the image of life in the leave, since it endures high temperatures, and saltiness when contrasted with numerous other fruit crop species. One of the most established relationships that man has had with a tree has been with date palms, which have been developed since ancient times (Zohary and Hopf, 2012). One of the character of date palm is that it can adapt to extreme drought, to heat, and to relatively high levels of soil salinity. Nevertheless, extreme quantities of salinity due to irrigation with saline water result to a significant decrease in the productivity of the fruits. It is important to study the mechanism of tolerance to these abiotic stress in order to develop future date palm varieties that can tolerate excessive soil salinity (Yaish & Kumar 2015).

Several insects invade date palm trees, of which the red palm weevil (RPW), *R. ferrugineus* (Olivier) (Coleoptera: Curculionidae), is a standout amongst the most essential and harming pests, being a noteworthy risk to date palm trees everywhere throughout the world. The red palm weevil is ordinarily well hidden, and numerous local pervasions have just recently been perceive, making the red palm weevil a pest of major economic significance in all Persian Gulf Countries. This study aimed to isolation and screening of antagonistic *B. bassiana* from diverse soils and date palm and plantation crop ecosystem in Hail region, Saudi Arabia.

MATERIAL AND METHODS

Soil samples, samples of infected and dead red palm weevil (RPW) insects and date plant materials collected according to RPW incidence map of Hail, Saudi Arabia during the period 2016-2017. The *B. bassiana* (BH-2) strain was isolated from soil sample collected from Al Koutha village in Hail region. About 500 grams of soil sample were placed in a plastic bag. Five larvae (L3-L4) of the red palm weevil *R. ferrugineus* were added to the soil substrate to be infected by the entomopathogenic fungi. The plastic bag was kept for 2-3 weeks at room temperature (20-25°C) and a ca. 80% humidity level was ensured inside the bag by periodic water sprays. Dead larvae were collected, placed on wet filter paper in Petri dishes and put in an incubator at 25°C. The mycelium and spores that emerged on larvae were transferred directly onto growth medium (PDA – PotatoDextrose Agar) in Petri dishes and incubated at 25°C for 10 days; the isolates were then purified by repeated transplanting (Goettel and Inglis, 1997).

The *B. bassiana* (BH-1, BH-2 and VBM) isolates were obtained from naturally infected RPW adults collected in from Jubbah village in Hail region (Fig 1). To promote conidial growth, mycosed RPW adult cadavers were placed separately on filter paper soaked daily with water to achieve ca.100% RH inside Petri dishes. The petri dishes were incubated at room temperature (20-25C°).

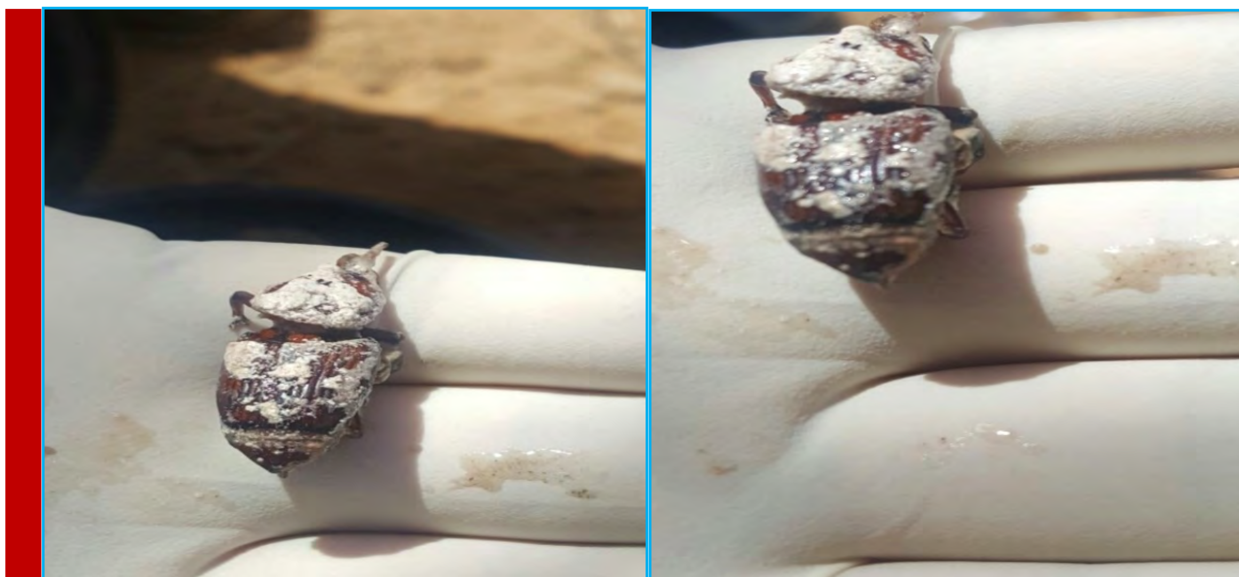

FIGURE 1. Fungal growth of red palm weevil in Hail region.

Parts of fungal propagules grown on the cadavers were then transferred, with sterile needles, into Petri dishes with SDAY1/4 (Sabouraud Dextrose Agar Fluka supplemented with yeast extract ¼of concentration) and kept in an incubator at 25°C. Pure fungal colonies were then stored on PDA (Potato Dextrose Agar) and MEA (Malt Extract Agar) slants in bacteriological glass tubes at 4°C. The *B. bassiana* isolates were confirmed by sequencing analyses of the 18SrRNA gene and the internal transcribed spacer (ITS1).

For microscopic identification of the fungus, pure fungal culture was used after successive purification. With the help of inoculating needle some portion of growth of the fungus was teased and place it on the slide then, 70% ethanol was used for washing, then ethanol was removed by blotting paper. Then, a drop of Lacto-phenol cotton blue was kept, and the mycelium was spread with needles, and a cover slip placed and examined under microscope with high power objective. The morphology and spore structures were noted.

API 20 C AUX kit was used to identify the strains of yeast isolated from RPW samples according to the manufacturer's instructions. Yeast strains were purified by culturing them on PDA (Potato Dextrose Agar) medium and incubated for 72 hours at 25°C. The strains were purified twice before identification. After 48 and 72 hours the growth was compared in each cupule to the 0 cupule, which is used as a negative control. A cupule more turbid than the control indicates a positive reaction to be recorded in the result sheet. Each strain was identified with the identification software by manually entering the 7-digit numerical profile via the keyboard.

The molecular analyses for the fungal isolates were performed at King Faisal University- the College of Agriculture and Food Science – Pest & Plant Disease Unit.

All isolates were re-grown on other PDA petri dishes and inoculated on Potato dextrose broth medium for microscopic examination (Fig 2), also for extraction of DNA and molecular identification.

DNA was extracted using modified method of Dellaporta and Hicks, 1983 as the following protocol: Twenty mgs of frozen- dried mycelium or fresh harvested mycelium were ground with Kontes pestles in a 1.5 ml tube with 500 µl of Dellaporta buffer (100 mMTris pH 8. 50 mMethylenediamine-tetraacetate EDTA, 500 mMNaCl, 10 mM beta mercaptoethanol (BME). Thirty three µl of 20% sodium dodecyl sulfate (SDS, w/v) were added, and incubate the mixture was vortexed and incubated for 10 min at 65°C.160µl of 5 M potassium acetate KoAc (Sigma chemicals) were added and vortexed. The mixture was spun for 10 min at 10,000 rpm in a microcentrifuge tube. 450 µl of supernatant were transferred to a new tube. 450 µl phenol, chloroform and isoamylalcohol (PCI) 25:24:1 were added and vortexed for 5min and then spun for 5 min at 10,000 rpm. 400 µl of the upper phase were removed to a clean micro-centrifuge tube and 0.5 volumes of isopropanol were added, vortexed and spinet for 10 min at 14,000 rpm. The supernatant was removed, the total nucleic acid was precipitated in the bottom of the tube. The pellet was washed with 70% ethanol and spun 5 min at 10,000 rpm. The pellet was resuspended in 100 µl of ddH2O.

Two primer pairs, the forward IT5 primer 5'-GGAAGTAAAAGTCGTAACAAGG-3') and the reverse ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') were used to

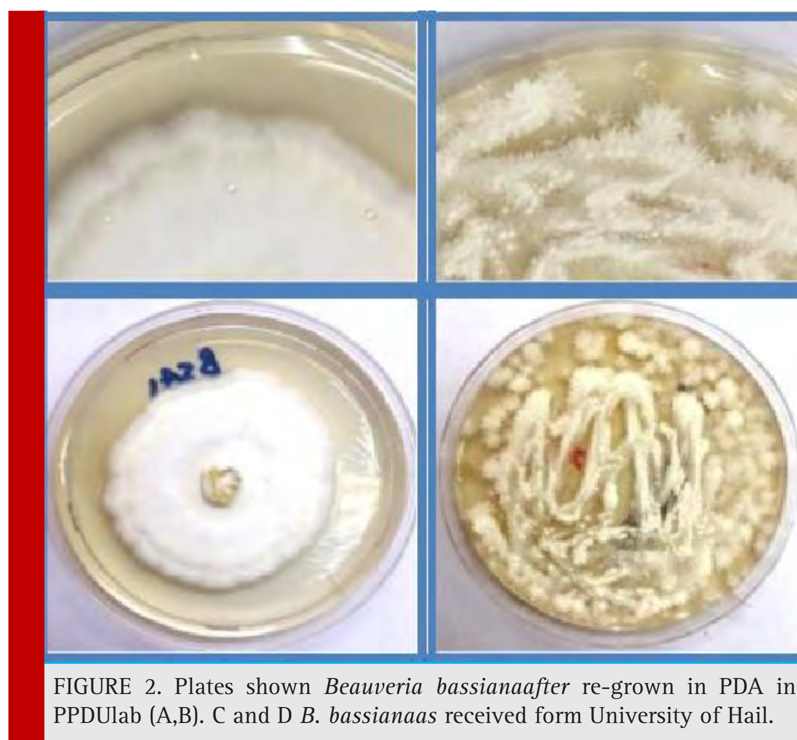

FIGURE 2. Plates shown *Beauveria bassianaafter* re-grown in PDA in PPDUlab (A,B). C and D *B. bassianaas* received form University of Hail.

amplify the entire ITS region (White *et al.*, 1990). PCR was done in a 25 µl reaction containing 1 µl of the fungal DNA extract (40 ng of total DNA), 2 mM MgCl2, 2.5 of 10x PCR buffer,1.5 µL of 10 µM of each primer, 2.5 µl of 10 mMdNTPs, 0.3 µl of 5U Taq DNA Polymerase and the reaction was completed to 25L with Nuclease-free water. PCR was conducted in the ESCO Swift Maxi Thermal Cycler with initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec, and the final cycle is a polymerization cycle performed at 72°C for 10 min. PCR Products were purified using QIAquick® PCR Purification Kit (Cat. No. 28106) according to manufacturing procedures.

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 MEGA6 (Tamura et al., 2013).

RESULTS AND DISCUSSION

Based on the API 20 AUX test performed on selected isolates of *Beauveria* spp. (Table 1), *Beauveria basiana* was the predominant yeast strain in all samples, also there was small percentage of *Aspergillus* spp. On the basis of the API 20 AUX test, the isolated yeast was identified as *Beauveria basiana*. Its profile makes up to 90% of the strains. As shown in the table, various assimilation profiles were obtained for *Beauveria basiana*. Studies on assimilation profiles were based on the acidification of

Table 1. The biochemical profile of *Beauveria* spp.

Sugar	Reaction	Sugar	Reaction	Sugar	Reaction
esculin	-	Adonitol	+	salicin	+
D-arabinose	-	rhamnose	+	glycogen	-
dulcitol	-	Inositol	+	dextrose	+/-
D-xylose	-	Lactose	+	trehalose	+
raffinose	-	sorbitol	+	maltose	+
galactose	+	dextrine	+/-	sucrose	+
D-fructose	+	mannitol	-	dolicitol	-

Note: + = Positive reaction
+/- = Weak reaction
- = Negative reaction

twenty sugars. The results indicate that galactose, fructose, rhamnose, maltose, sucrose and trehalose were assimilated at high degree contrasted with esculin, arabinose, xylose, raffinose, mannitol, glycogen and dulcitol. Biochemical properties and specifically their use of sugars can be utilized to help the morphology and are valuable for recognizing species of *Beauveria*. Mugnai *et. al.* (1989) contemplated the intra- and interspecific variety of 32 isolates appointed to the genus *Beauveria*. They presumed that cultural characters were profoundly factor also, couldn't be utilized dependably to separate species.

Depending on cultural and microscopic examination, fungal isolates belonged to two genera *Beauveria* and *Aspergillus* as shown on Table (2).

Table 2. Fungal speices isolated from *R. ferrugineus* in Hail region.

No	Label	Colour	Fungus name
1	BSA-1	white	Beauveria bassiana
2	BH-1	brown	Aspergillus ssp.
3	BH-2	white	Beauveria bassiana
4	BH-3	white	Beauveria bassiana
5	VBM	green	Aspergillus ssp.

All extracted DNAs from the three fungal isolates gave a clear bands on the expected size ≈ 600 bp using primer pairs ITS5&ITS4 (Fig 3). Blast analysis revealed that the fungal isolates are *Beauveria bassiana*. Sequences alignment (Fig 3) showed that all isolates had same sequence with 100% similarity with chinses isolates (Accession No. JQ320361). All sequences of the three isolates were deposited in the Genbank. 16 S rDNA nucleotide sequence has been sent to the Genbank for sequence publication.

The red palm weevil *R. ferrugineus* is a standout amongst the most serious pests of various date palm species, including date palms (Giblin-Davis, 2001). The weevils create inside the tree trunk, wrecking its vascular system and in the long run causing the crumple and death of the tree. The pest is generally circulated in Oceania, Asia, Africa and Europe. The RPW makes extreme harm to coconuts in Southeast Asia (Giblin-Davis, 2001). It showed up in the Middle East in the 1980s and has vigorously harmed date production by pulverizing huge number of date palms (Murphy and Briscoe 1999). Invasion was first reported in Israel and Jordan in 1999 (Khan and Gangapersad 2001).

The enormous economic losses created by RPW for date palm trees in Hail region encouraged the authors

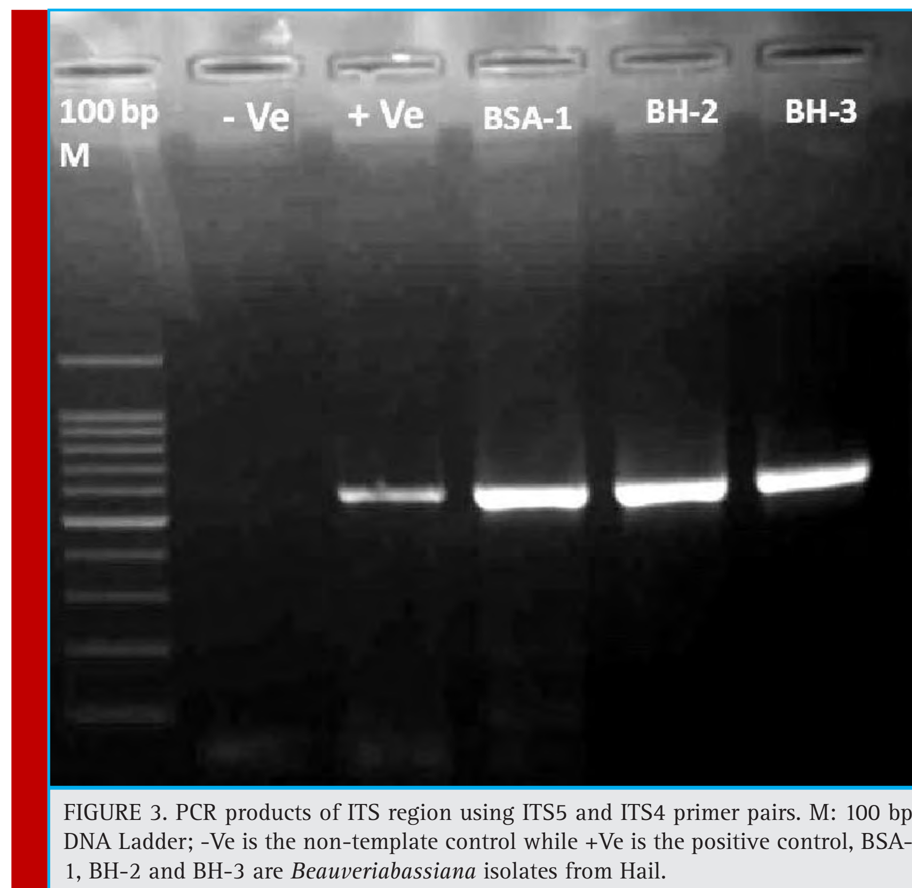

FIGURE 3. PCR products of ITS region using ITS5 and ITS4 primer pairs. M: 100 bp DNA Ladder; -Ve is the non-template control while +Ve is the positive control, BSA-1, BH-2 and BH-3 are *Beauveriabassiana* isolates from Hail.

to seek for an economically feasible technique to be employed for eradicating this harmful insect. During surveying the various infected date palm farms, many dead RPW were found under the infected trees covered with whitish material. In addition, farmers informed the researchers that they do not use any chemical treatment for pest control in their farms. This information motivated the researchers to try to recognize the causes of RPW death. Hence, the research has been resumed by collecting the dead insects are identifying the whitish material covering their bodies. The morphological tests indicated that the whitish materials were fungus growth. Subsequently, the biochemical identification tests indicated that the fungus was *Beauveria bassiana*. Then, the identification was confirmed using molecular biology tests.

Fungi are the commonest reason of insect disease in nature. Certain species of entomopathogenic fungi shown specificity to certain host (Wang, Chengshu & Wang, Sibao. 2017). Fungi may infect insects by direct infiltration of the cuticle thus works as contact insecticides. *Beauveria* is standout amongst other known genera of entomopathogenic fungi and worldwide various enrolled mycoinsecticide formulations in view *Beauveria bassiana* is utilized for control of insect pests (Thomas and Read, 2007). This fungus has an especially wide host range (over 700 species) enabling it to be utilized against vectors of human disease and an extensive variety of insect pests (de Faria, and Wraight, 2007). For instance in China, around one million hectares a year are treated with *Beauveria bassiana* to control forest insects such as the pine caterpillar *Dendrolimus punctatus* (Wang *et al.,* 2004)

Beauveria is outstanding for creating huge cluster of biological active secondary metabolites including non-peptide pigments and polyketides (e.g., *oosporein, bassianin* and *tenellin*), nonribosomally synthesized peptides (e.g., *beauvericin, bassianolides* and *beauveriolides*), and discharged metabolites associated with pathogenesis and destructiveness (e.g., oxalic acid) that have potential or acknowledged modern, pharmaceutical and agricultural uses (Xu *et al*., 2009). The mechanism of action and biological function of Oosporein have persisted unclear. *B. bassiana* has developed the ability to parasitize insects. A unique zinc finger transcription factor, BbSmr1 (*B. bassiana* secondary metabolite regulator 1), was identified in a screen for oosporein overproduction. Deletion of *Bbsmr1* resulted in up-regulation of the oosporein biosynthetic gene cluster (*OpS* genes) and constitutive oosporein production, (Fan *et al*., 2017).

The dominant part of endophyte explore has concentrated to date on the vertically-transmitted endophytes inside the genus *Neotyphodium* (Clavicipitaceae) that systemically colonize the over the ground parts of a few grasses. These clavicipita-ceous endophytes are generally known to present a variety of potential advantages to their grass host plants (Kuldau and Bacon, 2008). Less consideration has been given to the evenly transmitted non-clavicipitaceous endophytes, which are in nature and commanded by the Ascomycetes (Arnold and Lutzoni, 2007); of which a few genera are fungal ento-mopathogens (Ascomycota: Hypocreales).

Rising as an energizing new area of research, 'fungal ento-mopathogens as endophytes' has just rather recently been incorporated into a more than 100 old endophyte research base after the recuperation of different genera of fungal ento-mopathogens as endophytes from various plant species (Vega et al., 2008). Some of these fungi have been accounted for as normally happening endophytes, while others have been brought into the plant utilizing distinctive different inoculation techniques. Spearheading take a shot at entomopathogenic endophytes was directed with *B. bassiana* (Balsamo), a universal soil-borne fungus that infects an extensive variety of various insects (>700 insect species; Inglis *et al*., 2001) and is a standout amongst the most marketed fungal biopesticides (de Faria and Wraight, 2007).

Lewis and Cossentine (1986) credited the season-long concealment of the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) in maize *Zea mays* L. (Poaceae), estimated as reduced tunneling by the insect, to the establishment of *B. bassiana* as an endophyte following utilization of a watery suspension of the fungus to the plants. Ensuing work by Lewis and colleagues utilizing a similar model system demonstrated fruitful re-isolation of *B. bassiana* from interior plant tissues after utilization of the fungus utilizing different inoculation methods. These antagonist funfı can have beneficial effects on host plants, e.g., plant growth promotion, reducing disease severity, inducing plant defense mechanisms, and producing anti-herbivore products (Arnold and Lewis, 2005, Amatuzzi, *et al*., 2017).

In addition to maize, a wide assortment of host plants (counting both agronomic and weed species) have additionally been appeared to harbor *B. bassiana* as an endophyte. As opposed to *B. bassiana*, the host plant scope of other fungal entomopathogens is as yet developing. For example, *Verticillium (=Lecanicillium) lecanii* (Zimm.) Viegas has been accounted for as a natural endophyte in bear-berry *Arctostaphylos uva-ursi* L. (Ericaceae) (Widler and Muller, 1984) and ironwood (Bills and Polishook, 1991). A relatively recent development, the use of some fungal entomopathogens could function as biofertilizers. Various inoculation techniques (e.g., foliar sprays, soil drenching, seed soaking, injections, etc.) are effective in introducing fungal entomopathogens as

endophytes, but colonization appears to be localized and ephemeral, (Vega 2018).

There is presently considerable confirmation that some endophytic fungal entomopathogens, especially *B. bassiana* and *Lecanicillium* spp. (some time ago *Verticillium lecanii*), may likewise exhibit hostile action against plant pathogens , in addition to their outstanding biocontrol activity against insect pests. This proposes that these entomopathogens have a promising potential to be developed as biopesticides for numerous reasons in IPM methodologies (Goettel *et al*., 2008; Vega *et al.,* 2009; Ownley *et al*., 2010). *Beauveria bassiana* strain 11–98, applied as a seed treatment, has been accounted to suppress damping-off caused by the soil-borne pathogens, *Rhizoc-tonia solani* Kuhn (Basidiomycota: Cantharellales) and *Pythium myriotylum* Drechsler (Oomycota: Pythiales), in tomato (Ownley *et al*., 2004; Clark *et al*., 2006) and cotton seedlings (Griffin, 2007; Ownley *et al*., 2008). Pre-treatment of cotton seedlings with the same *B. bassiana* strain likewise resulted in reduced seriousness of bacterial blight caused by *Xanthomonas axonopodis* pv. malvacearum (Xam) (Griffin *et al*., 2006; Ownley *et al*., 2008).

More recently, several strains of *B. bassiana* were found to fundamentally diminish the rate and seriousness of the Zucchini yellow mosaic virus (ZYMV; genus Potyvirus, family Potyviridae) in squash (Jaber and Salem, 2014) and downy mildew caused by *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni. (Oomycota: Peronosporaceae) in grapevines (Jaber, 2015) following foliar inoculation of plants with conidial suspensions of the tested strains.

CONCLUSION

Strains of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchn.) Sorokin (Hypocreales: Clavicipitaceae) have been isolated from wild *R. ferrugineus* populations (Lo Verde et al., 2015). *Beaveria bassiana* fungus could be used as one of the biological strategies in controlling red palm weevil (Hajjar *et. al*., 2014). Based on the morphological, biochemical and molecular biological techniques, *B. bassiana* was isolated and identified as the fungal strain for the first time in Hail region, Saudi Arabia in which this fungal strain can be utilized in the biological control of the red palm weevil *R. ferrugineus* which is considered as a destructive pest of date palms in this region.

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Effect of school bags on body mechanics among Saudi children

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ABSTRACT

It is well known that school bag has effects on the posture of early school students. Therefore, lack of data about the impact of school bags on body mechanics of children from Saudi Arabia.This study aimed to assess the effect of heavy school bag on the student's body mechanics at elementary school in Riyadh. We conducted this observational cross-sectional study by observing the posture, palpation meter (PALM), interview, and examining school bag content, of primary school students across the Riyadh. Total of 200 students was included in our study. Less than half of the students reported pain (41.5%). Students who reported pain (n=83) most of them had inappropriate school bag weight (85.5%), whereas (14.5%) school bag weight was appropriate and (67%) students had tilted in the shoulders. Our finding suggested that heavy school bags contributed to the presence of pain and shoulder tilt, whereas long duration of carrying school bag did not affect shoulder tilt. Thus, in future, education for parents must be conducted to make sure to prevent students from carrying bags exceeding the acceptable standard limit.

KEY WORDS: SCHOOL BAG, ELEMENTARY SCHOOL, POSTURE, PAIN

INTRODUCTION

It is well known that schoolbag has effects on the posture of early school students. Studies have revealed that the prevalence of musculoskeletal issues in school children due to heavy school bags is increasing; therefore, it has become a concern, (Chow *et al.* 2010, Shamsoddini, *et al.* 2010). Earlier studies have shown that carrying heavy school bags has harmful effects on the children's musculoskeletal systems such as increasing in postural sway, and trunk muscle activity levels, Dianat *et al.* 2013). Students cannot carry heavy loads due to their

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size and weight. Thus, carrying heavy school bags leads to schoolbag-related injuries for children, Watson *et al.*, (2003). According to doctors and physical therapists, the schoolbag weight should not exceed 10-15% of the students' body weight to avoid musculoskeletal problems, Several workers (Murphy *et al.*2007, Dockrell *et al.* 2013, Al-Saleem *et al.* 2016 and Dowshen, 2017) have found the prevalence of back pain among school students by evaluating the school bags weight about their weight. Out of 2567 school students, 1860 (72.46%) were carrying schoolbags more than the acceptable standard. The results have shown that female student's school bags are more substantial than male student school bags, and 42% of the school students reported back pain.

Body mechanics education depends on the researcher that assesses the effects of mechanical loading or stress on controlling and supporting the spine. The mechanism of loading could be compression, torque, bending, shear, and tension or a summation of these forces to perform functional tasks from the dynamic nature, Lieber *et al.* (2000). Excessive forces working on the spine can cause a dynamic deformation of the spine structure the human body can reduce pain stimulation by lowering mechanical stress thus contribute to pain management Lieber *et al.*2000 . The majority of the studies focused on the schoolbag weight as the most important factor affecting the musculoskeletal system, while there are also other factors such as duration and method of carrying schoolbags affecting the musculoskeletal system, (Dianat *et al.* 2013, Dockrell *et al.* (2015) and Patil *et al.* 2016).

Carrying the school bag over one shoulder makes the student lean to one side due to the extra weight which harms the student by causing back pain, shoulder and neck strain. If the school bag is placed in a wrong way over the shoulders, it could lead to back, shoulder and neck pain because the student tries to resist the weight's force that pulls backward by bending forward which causes compression to the spine. Also, carrying school bag incorrectly causes bad posture Pascoe *et al.* (1997). Dianat *et al.* (2014) reported that musculoskeletal symptoms prevalent for 307 primary school students were caused by school bags. Results have shown that the shoulders were most affected body region among the sample with (70%), then wrists/hands (18.5%), upper back (13.6%) and low back (8.7%), while no symptoms were noted for the lower limbs, (Dianat *et al.* 2013) .

The basic assumption based on the biomedical model is that the maturing spine cannot handle the mechanical load of the backpack sufficiently Reneman *et al.* (2006). A previous study has shown that head, cervical spine and shoulder alterations in posture throughout gait terminations in student due to load carriage. Carrying more than the standard limit for schoolbag weight has effects on the posture of the head, cervical spine, and shoulder throughout gait terminations. The posture of the head became more forward, while it affected the shoulder to be more rounded and tilted Reneman *et al.* 2014, Mwaka *et al.* (2014). Center of gravity has a significant effect on schoolbag weight and in consideration of proper carrying techniques. Different carrying bag techniques is one of the significant factor on posture and gait of students aged between 11 $\pm$ 13 years old, there is no difference in the lateral spinal deviation between two-strap backpack and students without the bag, Nevertheless, two-strap school bag reduced carrying stress on the student back, neck and shoulders, (Pascoe *et al.*1997, Skoffer *et al.* 2007, Mwaka *et al.* 2014). Moreover confirmed the musculoskeletal pain as result of the heavy backpack.

The literature has demonstrated the detrimental effects of carrying heavy school bags on school students. Studies have revealed that the prevalence of musculoskeletal issues in school students due to heavy school bags is increasing. There was a lack of data about the effect of school bags on body mechanics of children at Saudi Arabia. So, we this study aimed to assess the effect of heavy school bag on the student at elementary school body mechanics in Riyadh. In this study, we took in consideration factors that contribute in heavy bags and the duration of carrying the bag.

MATERIALS AND METHODS

This study was carried out in primary schools in Riyadh, Saudi Arabia between Augusts to November 2017. It was an observational cross-sectional study. The sample size (n=200) divided equally for each gender as 100 males and 100 females. The sample included primary school students between 9-11 years old (third grade to fifth grade). We excluded students with congenital deformity, history of accident, students younger than 9 years, and older than 11 years, obese students. Multistage cluster sampling was used, thus we divided Riyadh into regions or clusters.

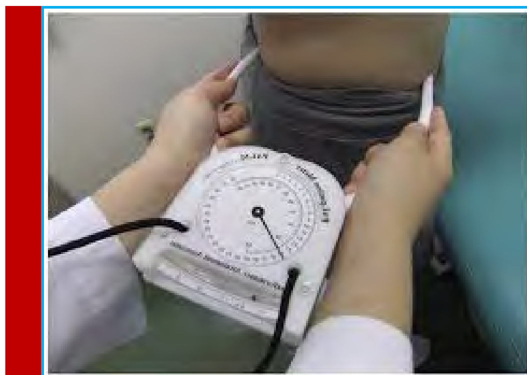
The schools were selected from eastern region, and students were chosen randomly. The participants in each school were waiting in a classroom and numbers were distributed to them randomly so we called them by the number then they came to do the assessment. One assessor measured the student weight and their bag weight, and examined student bags whether it was one/two strap, loose/tight, width of strap, and its contents. Second assessors were interviewed by self-designed questionnaire, and the other assessors were measured with Palpation Meter (PALM).

The questionnaire was developed to cover the areas we wanted to collect in this study. There were 22 questions about demographic details, pain, duration of carrying the school of bag, and whether they are educated about

the topic. The measurement tool that has been used in this study is valid and reliable tool called palpation meter (PALM) Petrone *et al.* (2003), Kellis *et al.* (2010). This tool allows to determine skeletal asymmetry and then locate the point of asymmetry by making measurements at various landmarks in the body. Also, we used scale to weigh the students and their bags. Before visiting schools, we contacted the school principal to permit the conduct of the study. Informed consent form was sent to the parents of the student for signed consent. All details of the study delivered to the parents before the start of the study.

DATA ANALYSIS

The calculated data entered into Microsoft Excel. Categorical variables represented in frequencies and percentage. Mean and Standard Deviation used for continuous variables. The data analysis used Chi-square test to compare proportion the difference for various variables. A statistical package was SPSS version 22 used for statistical analysis of the data. The P-value <0.05 was considered the level of significance. Appropriate diagrams have also been used.



RESULTS AND DISCUSSION

A total of 200 students from 4 different schools participated in the study. The average age was 9.58 years and the SD was 0.711. There were (100) 50% male and (100) 50% female. There were 110 (55%) third grade (9 years), 64 (32%) fourth grade (10 years), and 26 (16%) fifth grade (11 years). The study found that 83% of the students had inappropriate schoolbag weight and 17% appropriate as seen in (Fig 1).

It was found that female had more inappropriate school bag weight (54.2%), while this was 45% for students. The percentage of students with inappropriate school bag weight of each age group was highest for students with 10 years (90.6%), then 9 years (80%) and lowest in 11 years (76.9%) see Table 1. Students who were educated about the effects of school bag on body mechanics were less (32.5%) than who were not educated (57.5%). All the students had backpack and carried their backpacks over two shoulders and most of the bags had wide strap (91.5%) and loose strap (67.5%), whereas (8.5%) of their bags had thin strap and (32.5%) tight strap. The mean weight of school bags was 4.82 ±1.536 kg (Table 2). It was found that students who carried books according to their daily schedule were (84%) while (16%) did not. The majority of male students (66%) and female students (78%) had extra bag content. Less than half of the students reported pain (41.5%), and the rest (58.5%) of them did not. The students who reported pain (n=83) most of them had inappropriate school bag weight (85.5%), whereas (14.5%) school bag weight had appropriate school bag (Table 1).

The most area of pain reported was shoulder (70%), then (18%) in back. and the least reported was in their neck (12%). Among the students who reported pain, the majority felt pain while carrying the bag (79.5%), after carrying the bag (14.5%), and 6 % had felt it all the time

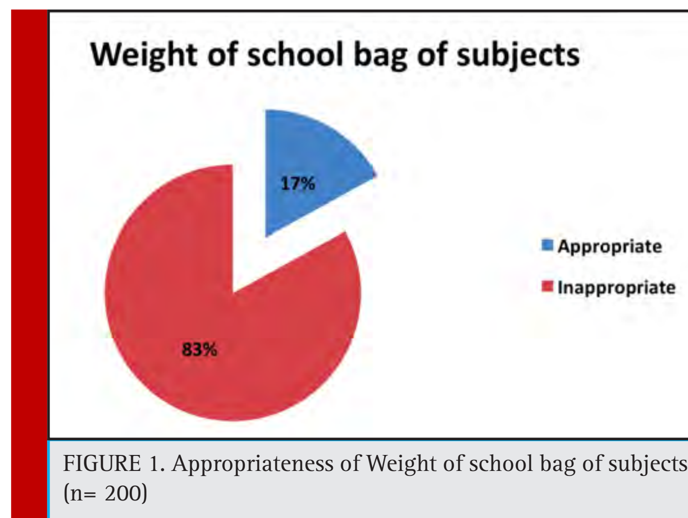


FIGURE 1. Appropriateness of Weight of school bag of subjects (n= 200)

Table 1. School bag weight effects					
				Chi square	P-value
		Appropriate	Not appropriate		
When do you feel the pain? (n=83)	While carrying the bag	7(58.3%)	59(83.1%)	9.224	0.010
	After carrying the bag	2(16.7%)	10(14.1%)		
	All the time	3(25.0%)	2(2.8%)		
What is the level of your pain? (n=83)	Mild	2(16.7%)	36(50.7%)	12.813	0.002
	Moderate	5(41.7%)	30(42.3%)		
	Severe	5(41.7%)	5(7%)		
Gender (n=200)	Female	5(20%)	46(42.2%)	4.252	0.039
	Male	20(80%)	63(57.8%)		
Educated about this topic before (n=200)	Yes	13(38.2%)	52(31.3%)	0.614	0.433
	No	21(61.8%)	114(68.7%)		
Tilt (n=200)	Yes	25(18.7%)	109(81.3%)	0.790	0. .374
	No	9(13.6%)	57(86.4%)		
Tilt side (n=134)	Right	17(68.0%)	69(63.3%)	0.195	0.659
	Left	8(32.0%)	40(36.7%)		
Where do you feel pain exactly? (n=83)	Neck	1(8.3%)	9(12.7%)	2.236	0.327
	Shoulder	7(58.3%)	51(71.8%)		
	Back	4(33.3%)	11(15.5%)		
Do you feel any pain? (n=200)	Yes	12(14.5%)	71(85.5%)	0.650	0.420
	No	22(18.8%)	95(81.2%)		
Age in years (n=200)	9	22(64.7%)	88(53.0%)	4.019	0.134
	10	6(17.6%)	58(34.9%)		
	11	6(17.6%)	20(12.0%)		

Table 2. The mean of school bag weight					
	N	Minimum	Maximum	Mean	Std. Deviation
Schoolbag Weight	200	2	14	4.82	1.536

(6%). Also, among those students who reported pain most of them had mild level of pain (46%), then moderate pain (42%) and very few had severe pain (12%). According to the age group, pain was highest among student who age 11 years (n=26) 13(50%) and lowest in 9 years (n=110) 41(37.2%). There was no significant difference between the prevalence of pain between male (49.4%) and female (50.6%). We found that carrying the bag in morning line increase the incidence of pain as (50%) who have answered yes to carrying it in the morning have pain whereas (3.87 %) who have not carry the bag in the morning line have pain see Table 3.

Students who had pain and tilt were 52 (26%), 19 (45.2%) of them were female, and 33 (80.5%) were male see Table 4. 134 (67%) students had tilt in the shoulders and 67 (33%) of them did not have any tilt. From the students who had tilt in their shoulders 19% had schoolbag weight appropriate while 81% had inappro-

Table 3. Carrying school bag during morning line * pain				
		Do you feel any pain?		Total
		Yes	No	
Do you carry your bag while standing in the school morning line?	Yes	22(26.6%)	22(18.8%)	44(22%)
	No	61(73.4%)	95(81.2%)	156(78%)
Total		83(100.0%)	117(100.0%)	200(100.0%)

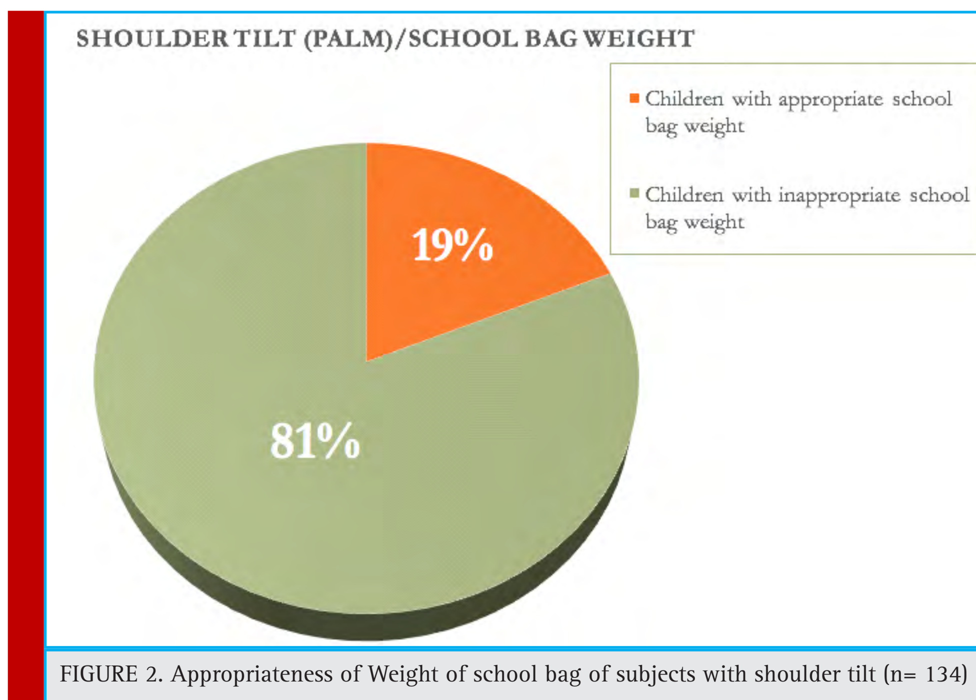


FIGURE 2. Appropriateness of Weight of school bag of subjects with shoulder tilt (n= 134)

priate bag weight (Figure 2). There was variety in the degrees of shoulders tilt from 1 to 4 degrees. Out of the students with shoulder tilt (n=134) the majority were male (61.9%), whereas (38.1%) of them were female. We investigated the link between the school bag weight and the presence of tilt in shoulders in each gender. It was found that out of the male with shoulder tilt (n=83) most of them carried school bag with inappropriate weight (75.9%), also in female (n=51) the majority of them carried school bag with inappropriate school bag weight (90.1%) see Table 1.

In this study, we investigated the school bag weight concerning the student body weight. 83% of the students had school bag weight exceeding the standard limit range (10%) the highest limit according to the Ireland Republic, European and Health Promotion Board of Singapore guidelines and only 17% had an appropriate bag weight, Al-Saleem *et al.* (2016). Alsaleem study found that 72.46% of their sample size had inappropriate school bag weight. Approximately the percentage was similar between our study and theirs, Al-Saleem *et al.* (2016). We found that female bags weight

Table 4. Gender comparison

Female 100 (50%)		Gender (n=200)		Chi square	P-value
		Male 100 (50%)			
Is the schoolbag weight appropriate (10% of the student's body weight) (n=200)	Appropriate	5(20%)	20(80%)	0.650	0.420
	Not appropriate	46(42.2%)	63(57.8%)		
Pain and tilt (n=83)	Yes	19(45.2%)	33(80.5%)	11.017	0.001
	No	23(54.8%)	8(19.5%)		
Tilt (n=200)	Yes	49(74.2%)	17(25.8%)	23.157	0.001
	No	51(38.1%)	83(61.9%)		
Bag Content (n=200)	Necessary things	22(22.0%)	34(34%)	3.571	0.059
	Extra things	78(78%)	66(66%)		

Table 5. Carrying school bag during morning line * tilt				
		Tilt		
		No	Yes	Total
Do you carry your bag while standing in the school morning line?	Yes	19(43.2%)	25 (56.8%)	44 (100.0%)
	No	47(30.1%)	109 (69.8%)	156 (100.0%)
Total		66(33%)	134 (67%)	200 (100.0%)

is slightly higher than male. Likewise, Alsaleem *et al.* results have shown that female student's school bags are heavier than male student's school bags. Also, a study found that female carried heavier schoolbags than male, Kellis *et al.* (2010).

The mean weight of school bags was 4.82 ±1.536 Kg, ranging from 2 kg to 14 kg, statistically significant at 0.05. All the students had a backpack and carried their back packs over two shoulders. Likewise, in Dockrell, Simms, and Blake study the mean schoolbag weight (4.8 ± 1.43 kg) and the majority (85%) carried their school bag over two shoulders. According to the age group, the percentage of students with inappropriate school bag weight was highest for students with ten years (90.6%). Inconsistent with another study, the relative bag weight was significantly heavier in the younger age group (ages 6–8 years), Kellis *et al.* (2010). Pain was highest among student with 11 years' students (n=26) 13 (50%) and lowest in 9 years (n=110) 41 (37.2%).

According to the measurement findings (PALM) we found that out of the students with shoulder tilt (n=134) 51(38%) of them were female while 83 (61.9%) of the male. We concluded that males have more shoulder tilt than female. Thus, the shoulder tilt correlated with the schoolbag weight (n=134), as a male with shoulder tilt and carried inappropriate school bag weight 63(57.8%) were more than female with shoulder tilt and carried inappropriate school bag 46(42.2%). The percentage of students who had shoulders tilt with inappropriate bag weight (81%) was higher than those with appropriate school bag weight (19%).this goes in line with the observations and recommendations of Dockrell *et al.*(2015) and Patil*et al.*(2016) .

The majority of male (66%) and female (78%) had extra bag items such as coloring books, and toys. In the Dockrell *et al.* study, it was also found that the students' bags had additional details such as sports items and musical instrument. These extra stuff increased the load carriage on students. Inappropriate school bag increased the incidence of pain and tilt in the shoulders. Carrying school bag during morning line increased the incidence of pain as most of the students who took their bags during morning line reported pain (50%). However, it did not show any evidence of increasing the shoulder tilt see Table 5.

The most area of pain reported was a shoulder (70%) this is matched the findings in Reneman *et al.*(2006), then (18%) in back. And the least reported was in their neck (12%). Similarly, in the study of Dockrell *et al.* (2015), the shoulders and the back were the most frequently reported areas of schoolbag-related discomfort. Students who reported pain (n=83) the majority felt pain while carrying the bag (79.5%), then after carrying the bag (14.5%), and the least had felt it all the time (6%). Students who reported pain (n=83) Most of them had a mild level of pain (46%), then moderate pain (42%) and very few had severe pain (12%) this matched the result of Spiteri *et al.* 2017. education for (32.5%) were made about the effect of heavy school bags on their body mechanics, but it was reflected negatively as we saw that (80%)of them had inappropriate school bag weight this indicated the poor response of the student to the education, may due to habitual attitude witch need long observation and correction by the family and the school teachers.

The students who had both pain and tilt were 52 students whom 19 (45.2%) female and 33 (80.5%) male students with a P-value 0.001. Thus, not every student with tilt was reported pain and vice versa. The presence of shoulders tilt was higher than the incidence of pain this goes in line with the findings of Ghousia *et al.* (2018).

Limitations of this study was it is only conducted in one region and Strengths of this study were that it considered other factors besides the bag's weight; the books should be unified from Ministry of Education for both public and private schools.

CONCLUSION

This study provided evidence that school bag weight has an effects on body biomechanics which causes shoulders tilt, and it also increased the incidence of pain. So, in future, education for parents must be conducted to make sure to prevent students from carrying bags exceeding the acceptable standard limit. The percentage of students with inappropriate school bag weight is high, so further research should investigate in depth the reasons for carrying heavy bags among primary school students.

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On the prevalance of selected serotypes of enterobacteriaceae pathogens isolated from polluted ecosystem

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ABSTRACT

Salmonella is a genus of rod shaped (bacillus) Gram negative bacteria of the family Enterobacteriaceae. *Salmonella* species are non-spore-forming, predominantly motile with cell diameters between 0.7 and 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella (all around the cell body). They are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. They are also facultative aerobes, capable of generating ATP with oxygen ("aerobically") when it is available, or when oxygen is not available, using other electron acceptors or fermentation ("anaerobically"). *Salmonella* species are intracellular pathogens; certain serotypes cause illness. Non-typhoidal serotypes can be transferred from animal-to-human and from human-to-human. They usually invade only the gastrointestinal tract and cause Salmonellosis, the symptoms of which can be resolved without antibiotics. Typhoidal serotypes can only be transferred from human-to-human, and can cause food-borne infection, typhoid fever, and paratyphoid fever. Typhoid fever is caused by *Salmonella* invading the bloodstream (the typhoidal form), or in addition spreads throughout the body, invades organs, and secretes endotoxins (the septic form). This can lead to life-threatening hypovolemic shock and septic shock, and requires intensive care including antibiotics. The present study is aimed at studying the Serotype of this Enterobacteriaceae pathogen isolated from sewage and drinking water environments.

KEY WORDS: ENTEROBACTERIACEAE, SALMONELLA, TYPHOIDAL SEROTYPE, SEWAGE AND DRINKING WATER ENVIRONMENTS

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INTRODUCTION

Salmonella species are facultative intracellular pathogens. A facultative organism uses oxygen to make ATP; when it is not available, it "exercises its option"—the literal meaning of the term—and makes ATP by fermentation, or by substituting one or more of four less efficient electron acceptors as oxygen at the end of the electron transport chain: sulfate, nitrate, sulfur, or fumarate **Cabada** *et al.*, **1975)**. Most infections are due to ingestion of food contaminated by animal feces, or by human feces. *Salmonella* serotypes can be divided into two main groups—typhoidal and non-typhoidal **(CDC, 2005)**.

Non-typhoidal serotypes are more common, and usually cause self-limiting gastrointestinal disease. They can infect a range of animals, and are zoonotic, meaning they can be transferred between humans and other animals **(Cooke, and Wain, 2005)**. Typhoid fever caused by *Salmonella* serotypes are strictly adapted to humans or higher primates—these include *Salmonella* Typhi, Paratyphi A, Paratyphi B, and Paratyphi C **(D'Aoust, 1989)**. In the systemic form of the disease, *Salmonellae* pass through the lymphatic system of the intestine into the blood of the patients (typhoid form) and are carried to various organs (liver, spleen, kidneys) to form secondary foci (septic form). Endotoxins first act on the vascular and nervous apparatus, resulting in increased permeability and decreased tone of the vessels, upset of thermal regulation, and vomiting and diarrhoea **(Hudault** *et al.*, **2001)**. In severe forms of the disease, enough liquid and electrolytes are lost to upset the water-salt metabolism, decrease the circulating blood volume and arterial pressure, and cause hypovolemic shock. Septic shock may also develop. Shock of mixed character (with signs of both hypovolemic and septic shock) is more common in severe Salmonellosis. Oliguria and azotemia may develop in severe cases as a result of renal involvement due to hypoxia and toxemia.

Mechanisms of infection differ between typhoidal and nontyphoidal serotypes, owing to their different targets in the body and the different symptoms that they cause. Both groups must enter by crossing the barrier created by the intestinal cell wall, but once they have passed this barrier, they use different strategies to cause infection. Nontyphoidal serotypes preferentially enter M cells on the intestinal wall by bacterial-mediated endocytosis, a process associated with intestinal inflammation and diarrhoea. They are also able to disrupt tight junctions between the cells of the intestinal wall, impairing the cells' ability to stop the flow of ions, water, and immune cells into and out of the intestine. The combination of the inflammation caused by bacterial-mediated endocytosis and the disruption of tight junctions is thought to contribute significantly to the induction of diarrhoea **(Murray, 1991)**.

Salmonellae are also able to breach the intestinal barrier via phagocytosis and trafficking by CD18-positive immune cells, which may be a mechanism key to typhoidal *Salmonella* infection. This is thought to be a more stealthy way of passing the intestinal barrier, and may, therefore, contribute to the fact that lower numbers of typhoidal *Salmonella* are required for infection than nontyphoidal *Salmonella* **(Olsen** *et al.*, **2001)**. Typhoidal serotypes can use this to achieve dissemination throughout the body via the mononuclear phagocyte system, a network of connective tissue that contains immune cells, and surrounds tissue associated with the immune system throughout the body **(Parras** *et al.*, **1984)**.

Salmonellosis is also known to be able to cause back pain or spondylosis. It can manifest as five clinical patterns: gastrointestinal tract infection, enteric fever, bacteremia, local infection, and the chronic reservoir state. The initial symptoms are nonspecific fever, weakness, and myalgia among others. In the bacteremia state, it can spread to any parts of the body and this induces localized infection or it forms abscesses **(Popoff, 2001)**. The forms of localized *Salmonella* infections are arthritis, urinary tract infection, infection of the central nervous system, bone infection, soft tissue infection, etc.Infection may remain as the latent form for a long time, and when the function of reticular endothelial cells is deteriorated, it may become activated and consequently, it may secondarily induce spreading infection in the bone several months or several years after acute salmonellosis **(Silverman, 1979)**.

MATERIALS AND METHODS

By plate count method 1 ml of the sample was prepared and transferred to 9 ml of saline and was maintained as master dilution. From this (10^{-1} to 10^{-6}) dilutions were prepared and 1 ml of sample was poured to cool sterilized agar count plate and incubated at 37°C for 24 hours. Colony was counted by colony counter. Morphological study was achieved by microscopic observation of Grams staining, Motility test, Catalase test and Oxidase test. A small portion of suspected colony was streaked on medias such as Nutrient Agar, MacConkey Agar and Eosin Methylene Blue Agar. Biochemical tests were performed using Standard Protocol. Following this serological typing was done. Depression plates were taken and were marked as A, B and C. In A depression plate it was marked as negative control in which phenolized saline suspension was added. In B depression

Table 1. Biochemical Test for Salmonella paratyphi A		
S. No.	**Biochemical Tests**	**Results**
1.	Catalase	Positive
2.	Oxidase	Negative
3.	TSI Test Butt Slant Gas H2S	Acid Acid Negative Negative
4.	Indole	Negative
5.	Methyl Red	Positive
7.	Voges Proskauer	Positive
8.	Citrate	Positive

plate it was marked as test in which phenolized saline suspension and antiserum of respective organism was added and in C depression plate it was marked as positive control which contain phenolized saline suspension of known organism and antiserum.

RESULTS AND DISCUSSION

The total number of positive and negative samples obtained from sewage and drinking water for *Salmonella paratyphi A* was found to be 13. In identification of bacterial isolate of morphological characteristics by Grams staining and motility for *Salmonella paratyphi A*, it wasfound to be Gram negative small rods and motile. Cultural characteristics of *Salmonella paratyphi A*on Nutrient Agar formed large transparent colonies, on Mac Conkey Agar lactose fermenting colonies and on Blood Agar moist colonies. Biochemical Test, Antibiotic Sensitivity Test and Serotype study results indicate the prevalence of *Salmonella paratyphi A* in the sewage and drinking water samples collected for the present study (**Tables 1 – 4 and Figures 1 – 8**).

Table 2. Antibiotic Sensitivity Test for Klebsiella pneumoniae			
S. No	**Name of the Antibiotics**	**Zone of inhibition in mm**	**Interpretation**
1.	Amikacin (AI)	13 mm	Resistant
2.	Chloramphenicol (C)	21 mm	Sensitive
3.	Co – trimoxazole (CT)	13 mm	Resistant
4.	Tetracycline (T)	20 mm	Sensitive
5.	Gentamycin (G)	14 mm	Intermediate
6.	Ceftriaxone (CTR)	22 mm	Sensitive
7.	Cephotaxime (CTX)	No zone	Resistant
8.	Norfloxacin (NX)	18 mm	Sensitive
9.	Meropenem (MR)	No zone	Resistant
10.	Imipenem (I)	No zone	Resistant
Zone of inhibition Below 10 mm – least active Between 11-25 mm – active Above 26 mm – very active			

Table 3. Serotyping of Salmonella paratyphi A from Sewage Sample					
S. No.	Name of the Isolate	Antiserum			
		O Antigen			H Antigen
		1	2	12	Phase 1 – a
1.	*S. paratyphi A – 1*	-	+	-	-
2.	*S. paratyphi A – 2*	-	-	+	-
3.	*S. paratyphi A – 3*	-	+	-	-
4.	*S. paratyphi A – 4*	-	-	-	+
5.	*S. paratyphi A – 5*	+	-	-	-
6.	*S. paratyphi A – 6*	-	-	+	-
7.	*S. paratyphi A – 7*	-	-	-	+
8.	*S. paratyphi A – 8*	-	-	-	+

Table 4. Serotypying of *Salmonella paratyphi* Afrom Drinking Water Sample

S. No.	Name of the Isolate	Antiserum			
		O Antigen			H Antigen
		1	2	12	Phase 1 – a
1.	*S. paratyphi A – 1*	-	+	-	-
2.	*S. paratyphi A – 2*	+	-	-	-
3.	*S. paratyphi A – 3*	-	-	-	+
4.	*S. paratyphi A – 4*	-	-	+	-
5.	*S. paratyphi A – 5*	-	-	-	+

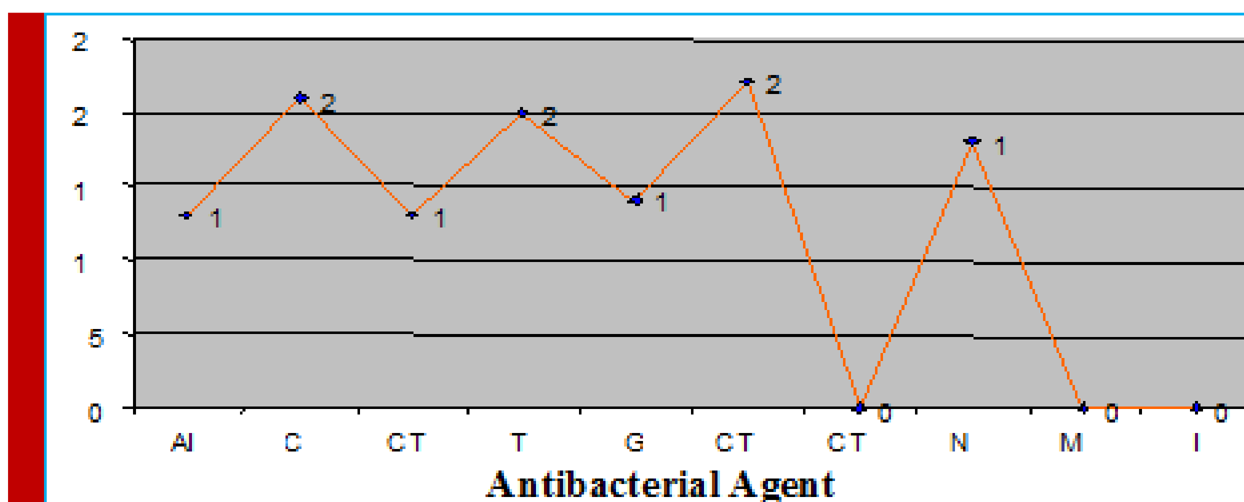

FIGURE 1. Antibacterial Activity of *Salmonella paratyphi* A. AI - Amikacin, C - Chloramphenicol, CT - Co-trimoxazole, T - Tetracycline, G - Gentamycin, CTR - Ceftriaxome, NX - Norfloxacin, MR - Meropenem, I – Imipenem

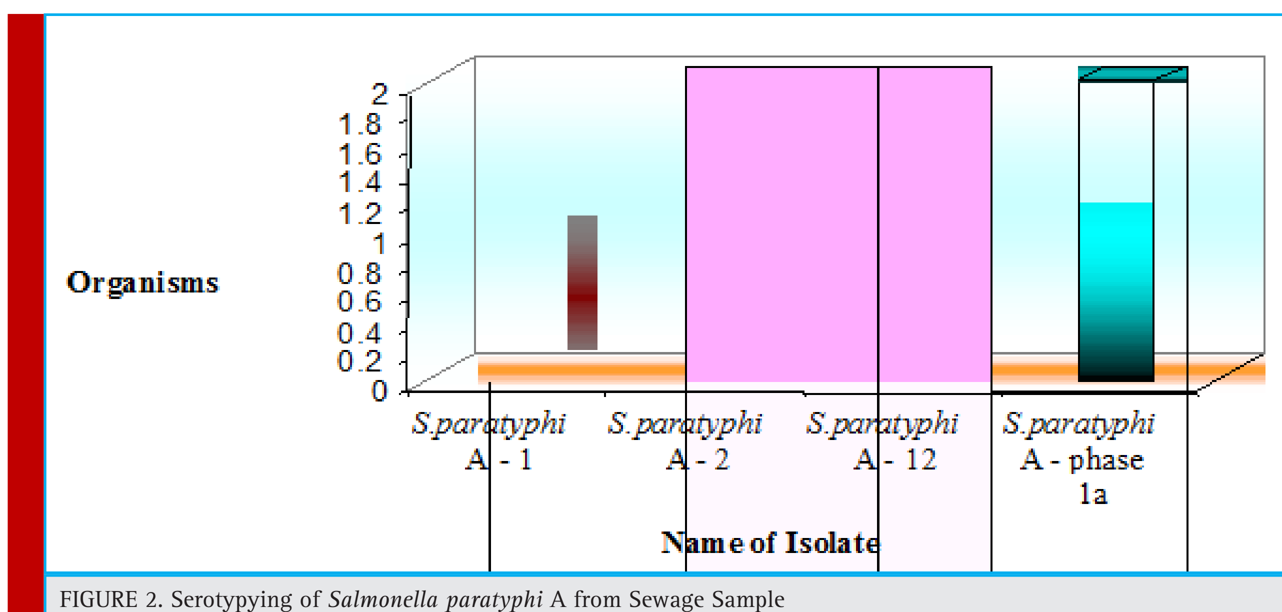

FIGURE 2. Serotypying of *Salmonella paratyphi* A from Sewage Sample

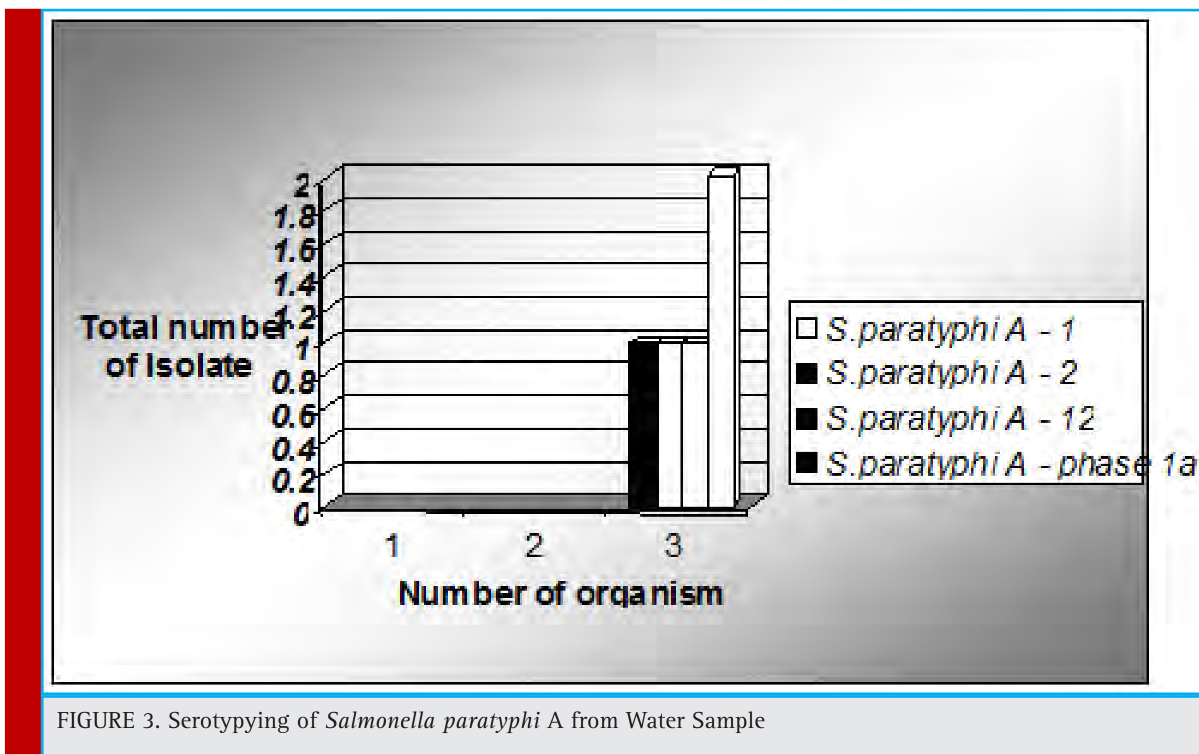

FIGURE 3. Serotypying of *Salmonella paratyphi* A from Water Sample

Salmonella infection (salmonellosis) is a common bacterial disease that affects the intestinal tract. Salmonella bacteria typically live in animal and human intestines and are shed through feces. Humans become infected most frequently through contaminated water or food. Typically, people with salmonella infection have no symptoms. Others develop diarrhea, fever and abdominal cramps within eight to 72 hours. Most healthy people recover within a few days without specific treatment. In some cases, the diarrhea associated with salmonella infection can be so dehydrating as to require prompt medical attention. Life-threatening complications also may develop if the infection spreads beyond your intestines. The risk of acquiring salmonella infection is higher if one travels to countries with poor sanitation. Thus the present study was aimed at studying this Enterobacteriacea pathogen which revealed high prevalence pattern in the water samples collected from polluted environments.

FIGURE 4. *S.paratypi* A on Nutrient Agar

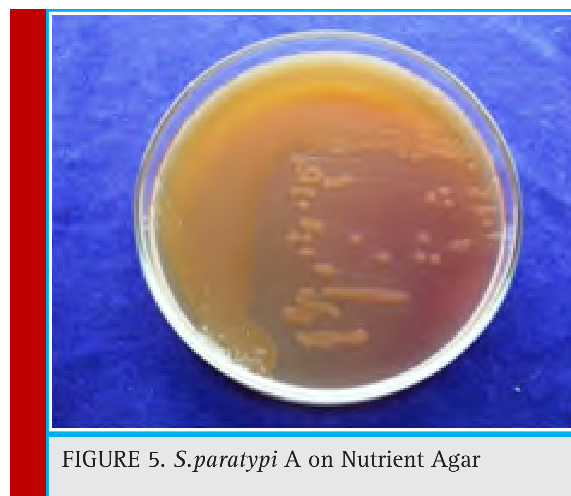

FIGURE 5. *S.paratypi* A on Nutrient Agar

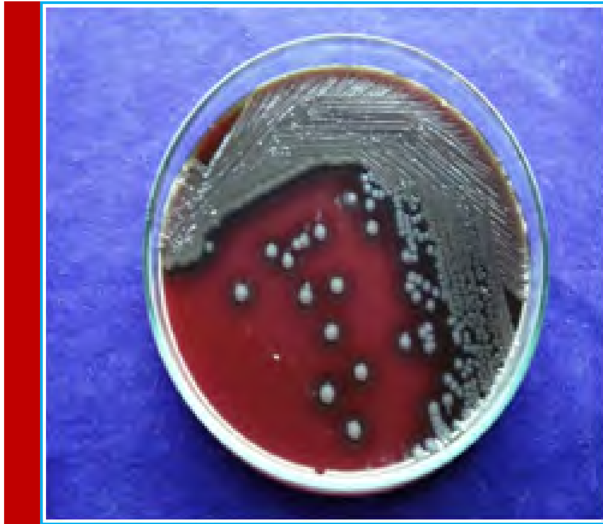

FIGURE 6. *S.paratypi* A on Blood Agar

FIGURE 7. IMViC test for *S. paratyphi* A. A = Indole B = Methyl Red C = Voges Proskaur. D = Citrate E = TSI

FIGURE 8. Antibiotic sensitivity test for *Salmonella paratyphi A*

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Enhanced production of alkaline protease from novel bacterium *Bacillus cereus* GVK21 under submerged fermentation

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ABSTRACT

Alkaline proteases are an important class of enzymes with potential industrial and commercial applications. In the present study, 52 bacterial isolates from various soil samples have been evaluated for the production of extracellular protease and selected one potent isolate based on maximum casein hydrolysis. Further, it is identified as *Bacillus cereus* GVK21 based on biochemical characteristics and 16S rRNA gene sequence analysis (KY659318). This organism produced 136 U/mL of protease within 48 hrs of incubation. Maximum production of protease was recorded at pH 9 and a temperature of 40°C. The present study is attempted to exploit new economical media, based on agro wastes recipes for the increased production of alkaline proteases. *B.cereus* GVK21 produced high levels of protease (1762 U/ml) on groundnut oil cake (1.5 %) as a substrate.The results of the study show that this isolate can be further exploited for commercial production of protease.

KEY WORDS: PROTEASE; *BACILLUS CEREUS*; 16S RRNA; OIL CAKES; INDUSTRIAL PRODUCTION

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INTRODUCTION

The emergence of new innovations are opening up new avenues in the areas of industrial biotechnology for the production of various bulk chemicals and value added products using inexpensive substrates (Binod *et al.*, 2013). In the recent years, enzymes are replacing chemical catalysts in food, leather goods, pharmaceuticals and textiles industry(Singh *et al.*, 2016). Proteases are a group of enzymes with wide range of applications, and account for 40 – 60% of the total enzyme sales with two thirds of them produced majorly from microorganisms (Kumar *et al.*, 2011; Deshmukh and Vidhale, 2015).

Proteases are highly complex group of hydrolytic enzymes and occupy a pivotal status with regard to their medicinal and industrial applications (El-Bakry *et al.,* 2015). Proteases are produced by a wide range of sources(Sharma *et al.,* 2017). The majority of commercially available enzymes are obtained from microbial origin (Raj *et al.,* 2012). Microbial proteases play a vital role in biotechnological processes and constitute one of the most important groups of industrial enzymes, selling product segment in the global market accounting for 60% market share (Kumar *et al.,* 2014).*Bacillus* genus is one of the most important producers of extracellular proteases and industrial sectors very often use *Bacillus* species for the production of proteases(Contesini *et al.* 2018).

In the recent years, bulk chemicals and value-added products such as organic acids, amino acids, enzymes, ethanol, and single cell protein etc., are produced by the utilization of agro-industrial residues as raw materials (Pandey *et al.*, 2000; Singhania *et al.*, 2008). Oil cakes or oil meals are the residues obtained after oil extraction from the seeds and are rich in proteins, fiber and energy contents (Ramachandran *et al.*,2007).The increase in the demand leads the attention of researchers to explore novel sources for proteases, where isolation, screening and characterization of new promising strains are a continuous process. Therefore, the present study focuses on the selection of high yielding stable proteolytic bacteria from mining soil sample which is considered as an extreme environment region.

MATERIALS AND METHODS

SAMPLE COLLECTION AND ISOLATION

Soil samples were collected in sterile containers from different locations within the mining area of Raichur district (16° 11' 45" North Latitude and 76° 38' 31" East Longitude), Karnataka, India. One gram of soil was added into100 mL of enrichment medium [g/L: Casein – 10.0 and NaCl – 5.0 at pH 9.0] in a 250 mL of Erlenmeyer flask and incubated at 37° C in a rotary shaker incubator at 140 rpm for 24 hours. A loopful of the enriched medium was streaked on Nutrient agar (NA) plates and incubated at 37° C for 24 hours. Well isolated colonies were re-streaked on NA for confirming the purity and transferred onto NA slants and preserved for further use.

SCREENING OF THE ISOLATES FOR EXTRACELLULAR PROTEASE PRODUCTION

The isolates were screened for protease production on screening medium, skimmed milk agar [g/L: skim milk - 10.0; peptone - 5.0; yeast extract - 3.0; NaCl - 5.0; agar, 20 and pH 9.0](Kumar *et al.,* 2011). After 24 hrs growth, the plates were observed for clear zones around the colonies. Proteolytic activity was further confirmed by using gelatin as the substrate in the medium (Pant *et al.*,2015). Depending on the zone of clearance, five isolates GVK8, GVK21, GVK29, GVK38 and GVK40, were selected for further experimental studies.

PROTEASE PRODUCTION UNDER SUBMERGED FERMENTATION

Protease production by the selected isolates was carried out by submerged fermentation. One mL of fresh bacterial inoculum was added in to 250 mL Erlenmeyer flask containing 100 mL of production medium [g/L: casein- 5.0, yeast extract - 5.0; NaCl - 5.0; $MgSO_4 \cdot 7H_2O$ - 0.02 and KH_2PO_4 - 0.05 at pH 9.0]. The flasks were placed in a rotary shaker incubator at 140 rpm at 37° C for 3-4 days. An aliquot of the culture supernatant was collected at regular time intervals of 12 hrs and assayedfor protease activity (Josephine *et al.*,2012). Based on the maximum protease production and shorter time required, the isolate GVK 21 is a potent strain and selected for further studies

IDENTIFICATION OF THE POTENT PROTEASE PRODUCING ISOLATE

Morphological and biochemical characteristics of the selected isolate (GVK21) was studied and recorded as per Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 2000). The bacterial isolate was further identified by 16S rDNA sequence analysis using universal primers and genomic DNA as template. The genomic DNA of the isolate was extracted as described by Roohi *et al.,* (2012). The PCR amplified product was sequenced at Microbial Ecology Laboratory, National Centre for Cell Science, Pune. Phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software using neighbor-joining method (Tamura *et al.*,2011). Duly annotated partial nucleotide sequences of the novel bacterial strain was deposited with NCBI Genbank (http://www.ncbi.nlm.nih.gov).

SCANNING ELECTRON MICROSCOPY

Scanning Electron Microscopic (SEM) analysis was done to observe the morphology of the isolated strain.Thin film of the sample was prepared on a carbon coated copper grid by just dropping a small amount of the bacterial culture on the grid. Extra solution was removed using a blotting paper. Then the film on the SEM grid was allowed to air dry by putting it under a mercury lamp for 5 min. The sample was then observed under scanning electron microscopy TESCAN (Vega 3 LMU) at a resolution of 3 nm at various magnifications at acceleration voltage of 20.0 KV.

PROTEASE PRODUCTION IN RELATION TO THE GROWTH OF BACTERIA

In order to study the time course for microbial growth and protease production, the isolate *B. cereus* GVK 21 was inoculated in the production medium and incubated in rotary shaker incubator at 160 rpm at 40° C upto 72 hours. The growth of the bacterium was determined by turbidometry, OD at 600 nm. After the removal of cells by centrifugation, the cell free supernatant was considered as the crude enzyme solution and protease activity was measured.

EVALUATION OF AGRO RESIDUES (OIL CAKES) FOR ENHANCED PROTEASE PRODUCTION

Major regional Oil cakes such as neem and pongamia were collected from local market, Bengaluru, Indiaand were fine powdered using mixer grinderand evaluated as substrates for the production of protease. Different type of oil cakes such as Castor oil cake (COC), Groundnut oil cake (GOC), Neem oil cake (NOC) and Pongamia oil cake (POC) were supplemented individually in to 100 mL of the optimized production mediumin 250 mL of Erlenmeyer flask. The agro wastes were used at a concentration of 0.5 to 2% with an increment of 0.25 %. The amount of protease produced was determined at every 6 h up to 72 h.

PROTEASE ASSAY

The culture was centrifuged at 10,000 rpm for 5 min at 4° C to obtain the CFS. The protease activity of the crude enzyme was determined by the modified method of Joo *et al.,*(2002) briefly; 0.5 mL of CFS was added to 0.5 mL of 1% casein (as a substrate) in 0.1 M Tris-HCl (pH 9.0) and incubated at room temperature for 10 min. The reaction was terminated by the addition of 3 mL of 10% (w/v) trichloroacetic acid (TCA). The solution was centrifuged at 5000 rpm for 10 min. To the 3 mL of the clear supernatant, 5 mL 0.4 M sodium bicarbonate solution and 0.5 mL of Folin Ciocalteau reagent (FCR) were added, mixed thoroughly and incubated for 30 min at room temperature, in dark. The optical density was measured using a UV-VIS spectrophotometer (ELICO SL-159) at 660 nm against the enzyme blank. The amount of the released aromatic amino acids was calculated usingtyrosine standard.

One unit of protease is defined as the amount of the enzyme required to release 1µg of tyrosine per mL per min under the above assay conditions. Enzyme activity was calculated according to the formula of Pant *et al., (*2015).

RESULTS AND DISCUSSION

ISOLATION, IDENTIFICATION, SCREENING AND CHARACTERIZATION OF PROTEASE-PRODUCING BACTERIA

In the present study, 52 independent bacteria were isolated from the soil samples collected from gold mines and were screened for proteolytic activity on skim milk agar plates (Figure 1). A total of 28 (53.84 %) isolates showed proteolytic activity ranging from 11 - 37 mm of clear zones aroundthe colonies. Five bacterial isolates, GVK8, GVK21, GVK29, GVK38 and GVK40, were selected based on the higher zone of hydrolysis on skim milk agar plates for further study. The use of skim milk agar medium for the efficient screening of proteolytic bacteria has been reported by earlier researchers (Raj *et al.,* 2012; Ravi *et al.,* 2015).

The selected five isolates were further screened for the quantitative production of the enzyme in production medium (Figure 2). The protease production was slower during the first 12 hours by all the isolates. The protease production increased significantly in respect of the isolate GVK21 during 12 to 24 hours to reach the maximum activity and remained at that level till 48 hours. While with respect to the other isolates, the protease activity increased gradually and the maximum activities were recorded at 48 hours of incubation. This indicated that the isolate GVK21 produces a maximum protease activity of 136 U/mL and takes shorter time in preliminary study.

The strain GVK21 is gram positive rod, motile, spore former and was tentatively identified as *Bacillus* sp. based on its morphological and biochemical characteristics (Table 1), (Holt *et al.,*2000). The 16S rDNA was amplified through PCR which showed1500 kb band on 2% agarose gel (Figure 3). Subsequently, the comparison of the 16S rRNA gene nucleotide sequence (1387 bp) of the strain GVK21 with other 16S rRNA genes sequences of closely related strains from NCBI database showed that this isolate has 99 % sequence homology with *B. cereus* ATCC 14579(Accession No. 074540). The

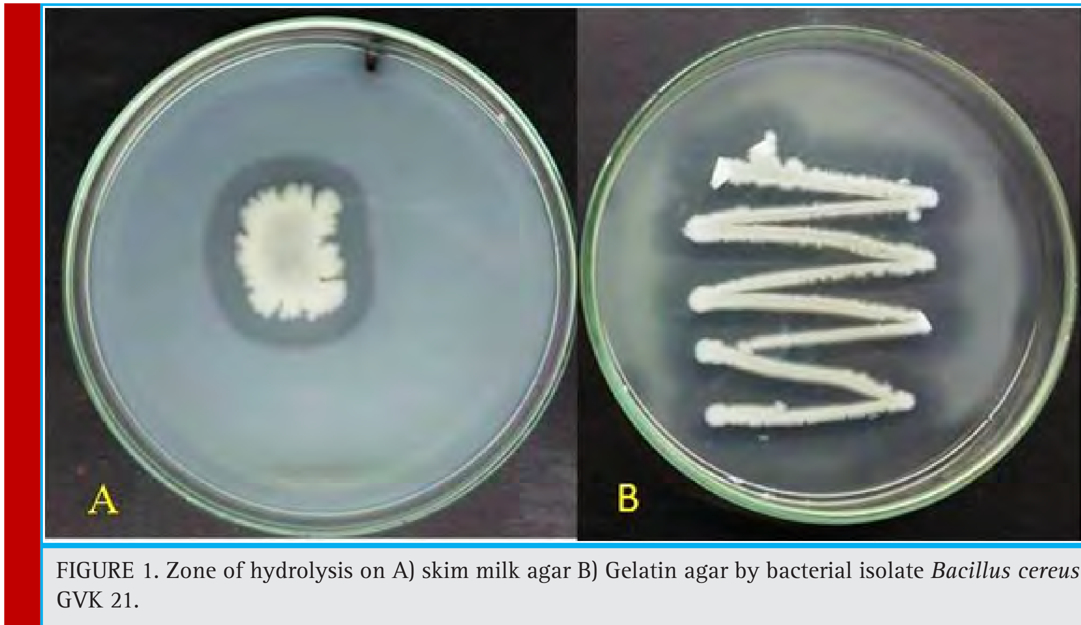

FIGURE 1. Zone of hydrolysis on A) skim milk agar B) Gelatin agar by bacterial isolate *Bacillus cereus* GVK 21.

phylogenetic tree, constructed by the neighbor-joining method indicated that the strain GVK21 is affiliated with the genus *Bacillus* and closely related to *B. cereus* strain LG1 - Accession No. KF307764 **(**Figure 4**).** The obtained nucleotide sequence GVK21 was submitted to GenBank database and the accession number assigned is KY659318 (https://www.ncbi.nlm.nih.gov/ nuccore/ KY659318). The species *B. cereus*ATCC 14579(074540.1), *B. cereus* strain JCM 2152 (113266.1), *B. thuringiensis strain NBRC 101235* (112780.1) has the closest sequence similarity of 99%.

Bacillus is an industrially important organism and was the first to be used in the commercial production of protease in 1952 (Binod *et al.*, 2013). Several pro-

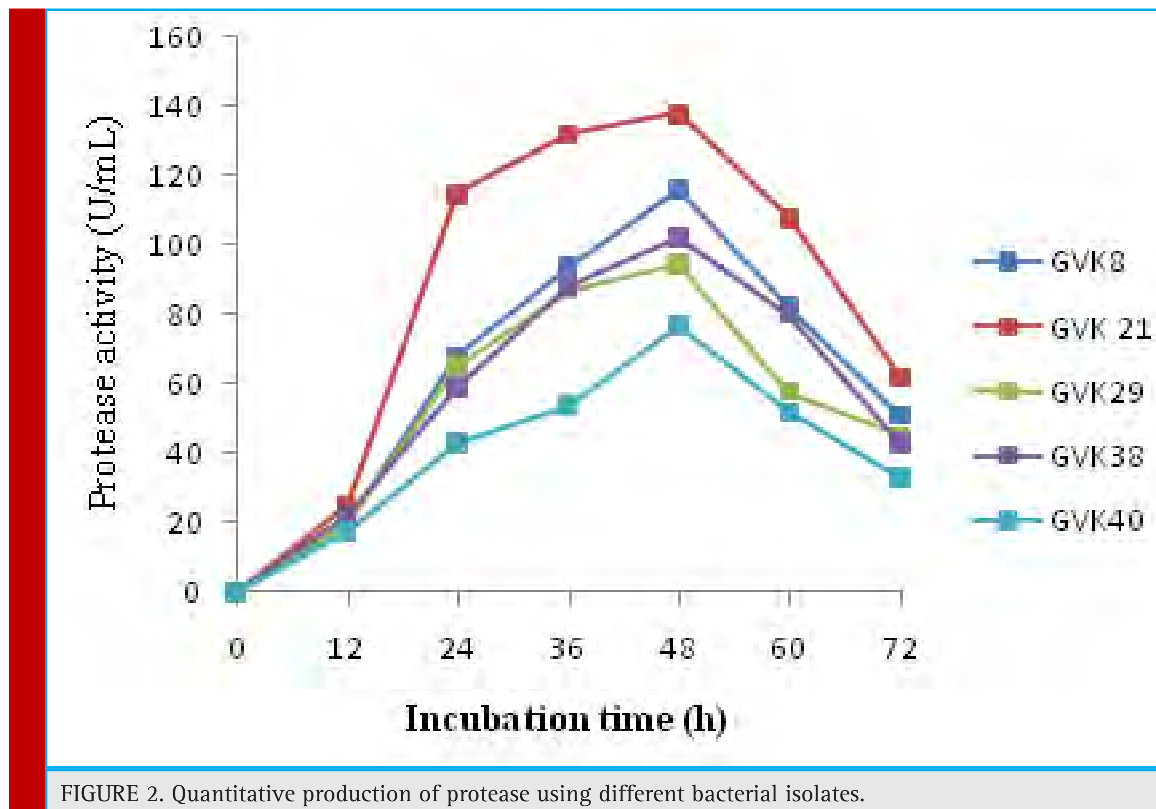

FIGURE 2. Quantitative production of protease using different bacterial isolates.

Table 1. Morphological and biochemical characteristics of *B. cereus* strain GVK21 isolated from soil.

Morphological Characteristics	Results
Gram staining	Positive rods
Colour	Creamish white
Motility test	Motile
Spore	Spore former
Physiological characteristics	
Catalase	Positive
Indole	Negative
Methyl red	Negative
Voges Proskauer	Positive
Citrate utilization	Positive
Oxidase reaction	Positive
Casein hydrolysis	Positive
Gelatin liquefaction	Positive
Starch hydrolysis	Positive
Nitrate reduction	Positive
Growth at 4o C	-
Growth at 40o C	+

teases have been produced from many *Bacillus* species (Rao *et. al.*, 1998). *Bacillus* species like *B. subtilis* (Babe and Schmidt, 1998), *B. licheniformis* (Mabrouk *et al.*, 1999), *B. sphaericus* (Singh *et al.*, 1999), *B. proteolyticus* (Bhaskar*et al.*, 2007), *Bacillus cereus* (Doddapaneni *et al.*, 2009; Bajaj *et al.*, 2013) *and B. megaterium* (Rajkumar*et al.*, 2010) have been reported for protease production. Other than *Bacillus* species bacteria such as *Pseudomonas fluorescens* (Kalaiarasi and Sunitha, 2009), *P. aeruginosa* (Raj *et al.*, 2012), *Vibrio etschnikovii* (Jellouli*et al.*, 2009), *V. alginolyticus* (Shanthakumari *et al.*, 2010) are also reported for protease production.

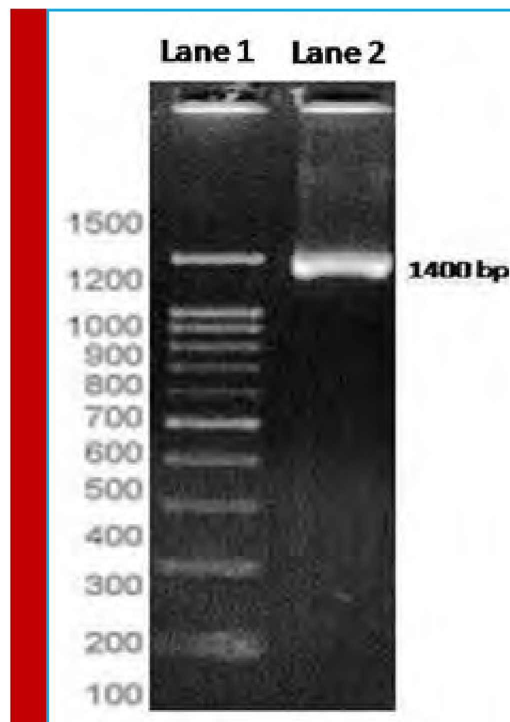

FIGURE 3. Agarose Gel Electrophoresis of *B.cereus* DNA. Lane1- 100 bp DNA ladder, Lane2- 16S rDNA amplicon of *B.cereus* GVK21

Bacillus cereus GVK 21 was subjected for secondary screening for quantitative protease production under submerged condition. The protease production increased significantly from 12 U/mL to 136 U/mL during6 h to 36 h of incubation and the enzyme activity was in synchrony with the growth of the bacterium, wherein the logarithmic growth was observed during 6 to 24 hours and the maximum growth was observed at 36 hours

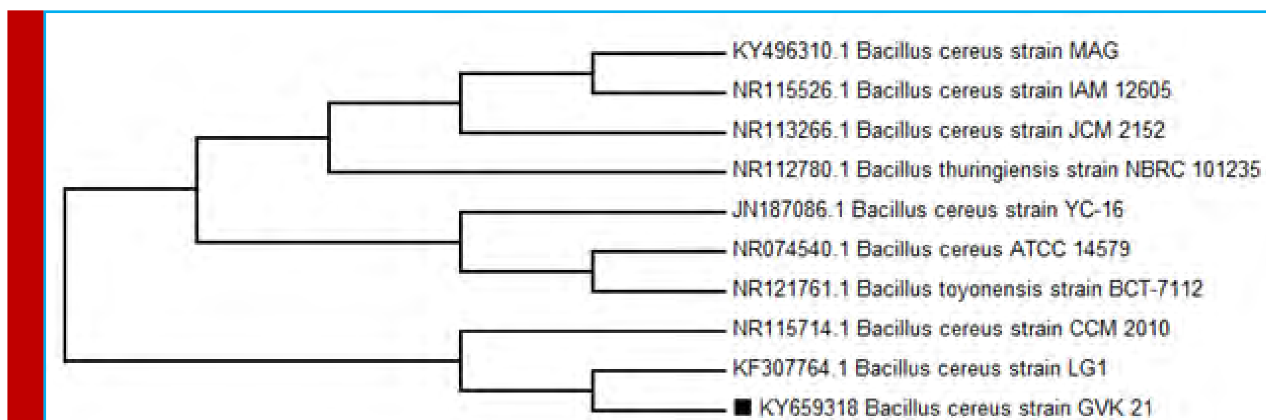

FIGURE 4. Phylogram obtained based on phylogenetic analysis of 16S rDNA gene sequence data showing the phylogenetic positions of isolate *Bacillus cereus* strain GVK21 and of a number of related taxa

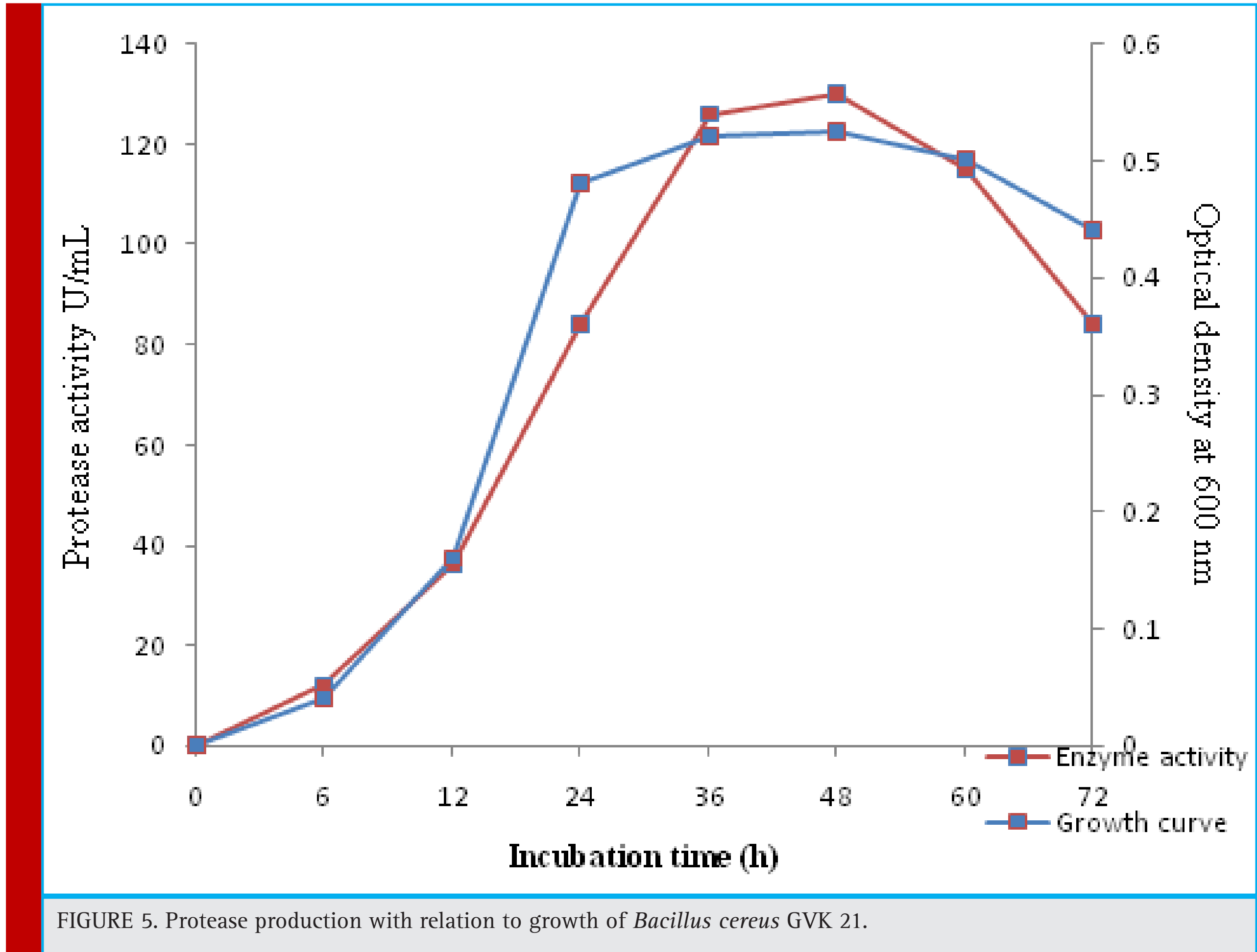

FIGURE 5. Protease production with relation to growth of *Bacillus cereus* GVK 21.

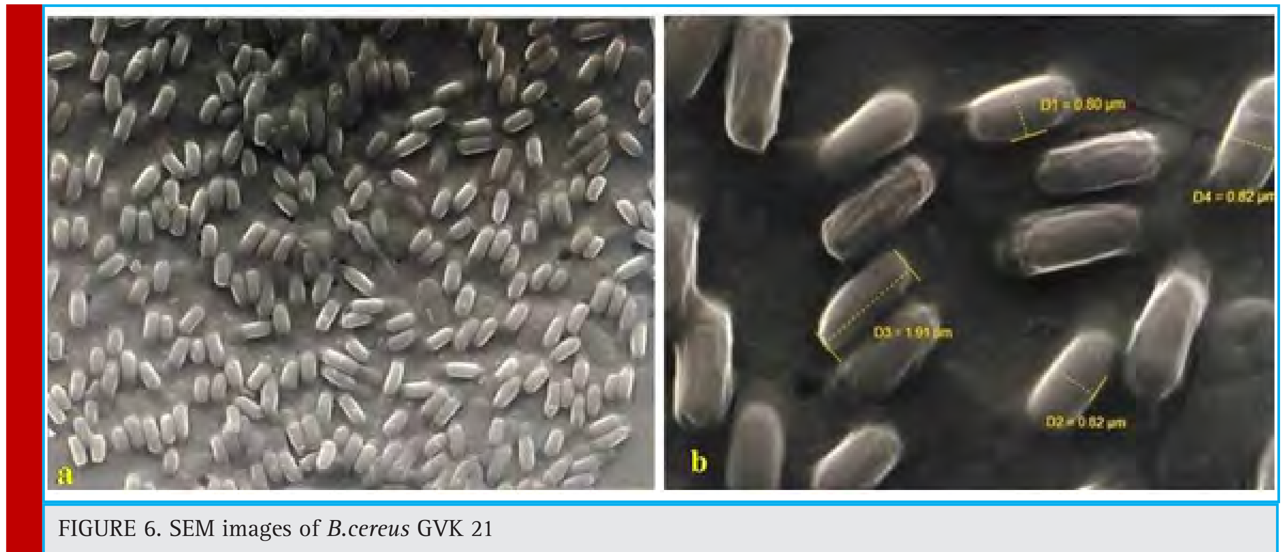

FIGURE 6. SEM images of *B.cereus* GVK 21

indicating that the protease production is maximum in the late exponential and early stationary phase (Figure 5). The decline in the protease production was observed after 48 h. Kannikar *et al.*,(2008) reported that *Bacillus* sp. BA40 produced 1.158 U/mL protease activity, *B. licheniformis* LBBL-11 showed 18.4 U/mL at 48 hours (Olajuyigbe, and Ajele,2008). The results indicate that the isolate *B.* cereus GVK 21 is producing 136U/mL a comparatively higher protease activity, that too under preliminary screening.

Chitte and Dey (2000) have also shown that the log phase the optimum for the production ofprotease by *Streptomyces megasporus*. Incubation time of 24 hours positively influences the production of protease from *Bacillus cereus* SRM-001 (Narasimhan *et al.*,2015). Incubation time of 40 h was found to enhance protease production by *Bacillus natto*-NRRL-3666 (Mahajan *et al.*,2010).

SEM ANALYSIS

Figure 6 shows SEM image of bacteria which confirmed the rod shaped nature of the cells, where the cell size (length and breadth) is found to be 1.91 µm and 0.82 µm respectively.

ENHANCED PROTEASE PRODUCTION USING OIL CAKES

The effect of agro-based by products as alternative substrates for bacterial protease production under submerged fermentation has been studied by several workers (Praveen Kumar *et al.*, 2008, Prasad, *et al.*, 2014). *B. cereus* GVK21 (KY659318) produced varying levels of alkaline protease on various agriculture based substrates. Among various oil cakes (Figure. 7) examined, groundnut oil cake enhanced the protease production from *B. cereus* GVK21 by 112 % (1762 U/ml) as compared to control (665 U/ml), i.e., groundnut oil cake supported maximum protease production. *Bacillus subtilis* SHS-04exhibited maximum protease production (1616.21 U/mL) by utilizing groundnut cake as substrate (Olajuyigbe, 2013).

Other oil cakes also substantially supported protease production, castor cake (1316 U/ml), pongamia cake (1144 U/ ml) and neem cake (904 U/ ml) as shown in Figure 8. Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium as also observed in the presence of ammonium sulphate and potassium nitrate (Saurabh *et al.*, 2007; Bajaj and

FIGURE 7. Different Oil cakes used as a substrate for protease production.

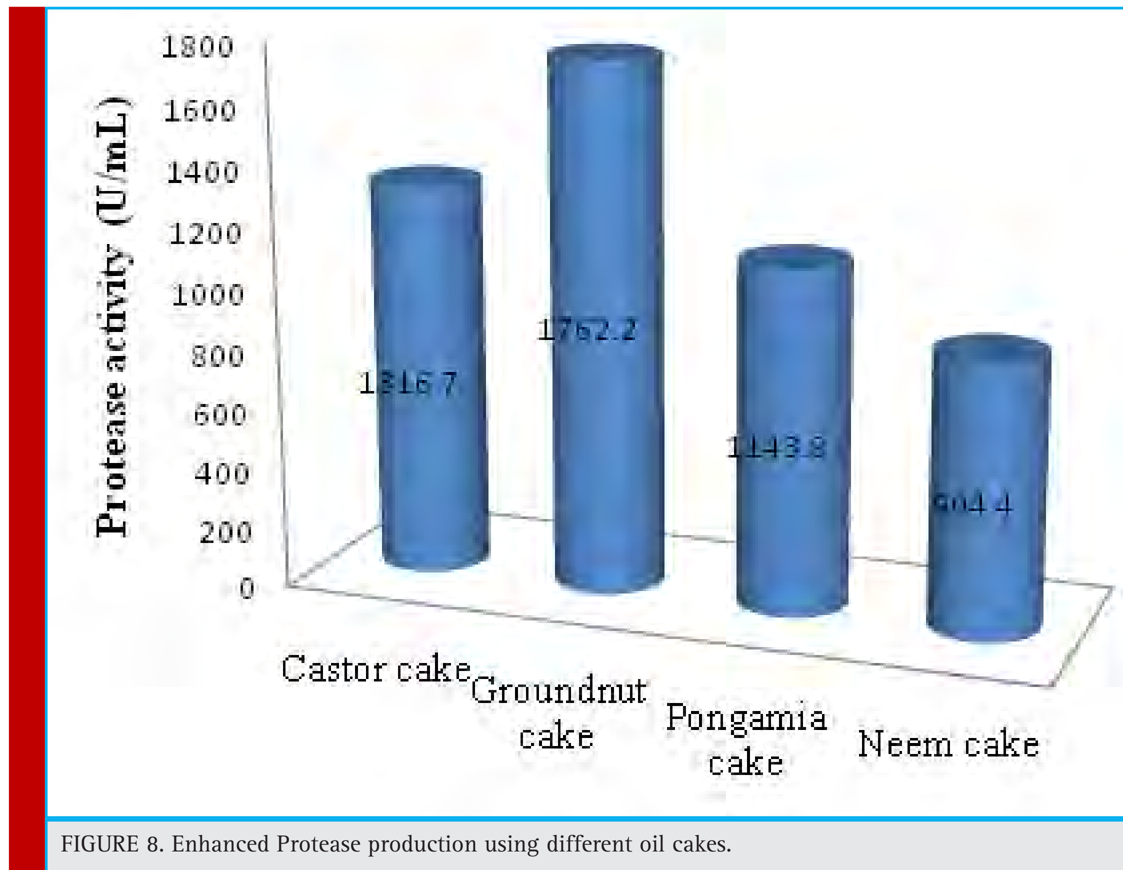

FIGURE 8. Enhanced Protease production using different oil cakes.

Sharma, 2011). Therefore complex nitrogen sources are usually used for protease production. Groundnut oil cake, a rich protein source (30–40%), which mainly constitutes different amino acids such as arginine (11.0), Leucine (6.1), glycine (6.0), and phenyl alanine (4.9) and a dry matter of 92.6% comprehends slow release of nitrogen due to its conditioned and moderately complex nature which may favor the process organism metabolically and physiologically for efficient production of protease (Kuo, 1967). Several reports indicate that groundnut cake serves as a reasonably good nitrogen source for protease production by various microorganisms (Kuberan *et al.*, 2010; Kranthi *et al.*, 2013, Olajuyigbe, 2013).

The substrate in the growth medium constitutes a major cost determining factor for the commercial production of industrial enzymes. The high cost of protease production is another major hindrance for wide range of industrial and medicinal applications of this enzyme (Jayasree *et al.*, 2009). Utilization of agro industrial residues as carbon and nitrogen sources for the bulk production of industrial enzymes may play a significant role not only in reducing the production cost and also contribute towards growing environmental concerns by addressing the agro industrial waste disposal and management problems because of tremendous quantities of agricultural residues generated through agricultural practices and industrial processes (Bajaj and Wani, 2011; Singh and Bajaj 2015). Earlier workers also reported that defatted oil meals like soybean meal (Saurabh *et al.*, 2007), cotton seed cake (Bajaj *et al.*, 2013) and groundnut meals (Olajuyigbe, 2013) are the cost-effective alternatives for industrial production processes. Hence utilization of a low-priced nitrogen source is an important criterion for economic production of industrial enzymes. Requirement of specific nitrogen source differs from one organism to other even among the same species isolated from different sources (Bajaj and Sharma, 2011).

CONCLUSION

Bacillus cereus GVK 21 (KY659318) isolated from the soil samples from a mining area exhibited higher proteolytic activity of 136 U/mL which significantly increased to 1762 U/mL on ground nut cake, comparatively higher than already reported and thus can be exploited as a potential source for large scale production of protease enzyme to cope up the needs of industrial applications and the demand of the global market.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest in the publication.

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Screening of indigenous active lactic acid bacteria isolated from freshly drawn raw milk

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ABSTRACT

Main emphasis of the present study was to isolate and identify active Lactic Acid Bacteria from various raw milk. Totally 36 freshly drawn various raw cow, buffalo, goat and sheep milk from Tirupur and Erode region in Tamil Nadu, were collected. From the sample 56 Lactic Acid Bacteria (LAB) isolates were taken randomly. The LAB were phenotypically identified and grouped based on the morphological, physiological and biochemical study. The strain survival were also assessed under stomach acid condition like low pH and resistance to bile salt 0.3%. Antibiotic sensitivity tests were performed for five antibiotics. After hemolytic activity on blood agar medium the antibacterial activities of the isolates were tested against two pathogenic bacteria *E.coli* and *Staphylococcus sp*., at pH 6.5 by overlay method. The tested isolates showed invitro inhibitory zone against pathogenic bacteria. From this study we can conclude that raw milk is good source of active lactic acid bacteria. Out of the 56 LAB isolates 28 exhibited good probiotic properties and potentiality was characterized in future.

KEY WORDS: RAW MILK, LACTIC ACID BACTERIA, PHENOTYPICAL IDENTIFICATION, ANTIBIOTIC SENSITIVITY, ANTIBACTERIAL ACTIVITY

INTRODUCTION

Ancient Indian has practice to consume freshly drawn raw milk without boiling. They believed that raw milk has some good property. Milk is an excellent medium to carry an active lactic acid bacteria and buffering capacity of milk helps to improve the survival of probiotic flora in the gastrointestinal tract, (Kailasapathy and Phillips 2008).Lactic acid bacteria tolerate high salt concentrations as its allows the bacteria to begin metabolism, which produces acid that further inhibits the growth of undesirable microorganisms (Pooja Thakkar *et al.,* 2015). Probiotic LAB can be a suitable alternate of antimicrobial agents and recently found to play a

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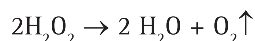
positive role in mental health. Milk is one of the natural habitats and rice source of LAB, (Delavenne *et al.,* 2012, Wouters *et al,* 2002 and Misganaw wassie and Teketay wassie, 2016, Mokoena *et al.,* 2016).

The LAB in milk and milk products enhance bioavailability of nutrients and act as a preservative(Misganaw wassie and Teketay wassie, 2016). Fermented and functional foods and the products are crucial to the human health(oktay Yerlikaya, 2014).Potential probiotic isolates of *Lactobacillus rhamnosus* and *L. plantarum* were present in indigenous goat milk (Setyawardani *et al.,* 2011). The probiotic *L.yoghurt* supplementation to worldwide waterborne diarrhea causing Giardia infected mice reduced the severity of Giardia infection (Geeta shukla *et al.,* 2010). Lactic acid bacteria produce antimicrobial compounds, vitamins or useful enzymes which could help in promotion of food industry (Ashmaig *et al.,* 2009). Identify potent attributes to meet out current demands of the functional food industry (Subhashini, 2014).

MATERIALS AND METHODS

36 samples of raw fresh milks were collected from lactating cow, buffalo, goat and sheep in the rural area surrounding of Tirupur & Erode District. Samples were collected using sterile centrifuge tubes and stored in an icebox until delivery of the laboratory for analysis. Till the analysis samples were kept in 4°C(refrigerator). About 1ml of milk sample was mixed with 9ml of saline [8.5g / L] to make an initial dilution [10^{-1}]. The suspension was used for making suitable serial dilutions up to 10^{-8}. Enumeration of LAB was determined using MRS (Man de Rogosa Sharpe) agar and M17 agar medium by pour plate [1ml in 15ml medium] incubated at 37 °C for 24-48 hours. After incubation colonies were chosen based on their morphology on MRS (pH-5.7) agar plate. The typical LAB were randomly picked up and purified for further work. Simple tests such as gram staining, catalase test, motility and sugar fermentation test were performed for isolates.

The isolates grown in freshly prepared liquid media and incubated overnight. After incubation the cells were taken and then gram staining procedure was performed. The gram reaction of the isolates was determined by light microscopy. Catalase enzyme produced by many microorganisms that breaks down the H_2O_2 into water and oxygen that releases O_2 gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.



$$2H_2O_2 \rightarrow 2\ H_2O + O_2\uparrow$$

The freshly grown liquid cultures were also used for catalase activity by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures and their catalase activity was observed.

(Thakkar *et al.,* 2015). MRS broth supplemented with different Sugars (glucose, lactose and maltose) and phenol red as pH indicator was inoculated with active cultures at 1%, incubated at 37°C for 24 hours. The cultures were identified based on acid and gas production in Durham's tube after the incubation period. To check the growth of isolates at various pH, MRS broth supplemented with different pH 2.0, 3.0, 7.0, 8.5 was prepared, 1% of fresh culture was inoculated and then incubated at 37°C for 28 hours. During incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth. Turbidity also measured at 620nm.Overnight active cultures were inoculated at 1% in MRS broth tubes and incubated up to 7 days at 15, 37, 45 and 55°C. Extent of growth was visually recorded based on intensity of turbidity. Overnight grown active cultures were inoculated at 1% in MRS broth tubes adjusted to various concentrations of Na Cl viz. 3.5, 6.5 and 18% (w/v) along with their respective controls. The cultures were incubated at 37°C. After 24 hours of incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

Blood hemolysis test was carried out as per the method of Mabrouk *et al.,*(2014). As the strains were isolated from food material, blood haemolysis test was performed, to eradicate any chance that our isolates may be pathogenic. It is also one of the criteria for assessing the safety of use of probiotics as food supplements. Pathogens produce highly toxic substance which lyse the RBC and forms a clear zone around them. The haemolytic activities of isolated strains were determined according to (Marakoudakis *et al.,* 2009) as follows: all examined strains were grown in MRS broth at 37°C for 24 hours and then streaked onto Columbia agar base plates supplemented with 5 % (v/v) whole human blood. The plates were incubated at 37 °C for 48 hours. Then the clear zones and the color of haemolysis around the growth colonies were observed. Antibiotic susceptibility test was done using the method of Singh *et al.,* (2014).

Probiotic strains must be sensitive to wards the antibiotics. There is a light risk that antibiotic resistance probiotic strain may transfer the antibiotic resistance genes to the pathogens via transformation in the gut. Due to any chance resistant pathogens get introduced into the human via food chain and cause serious problems. Sensitivity of probiotics strains towards the antibiotics being tested by using Kirby - Bauer disc diffusion technique. Tetracycline, Penicillin, Vancomycin, Strep-

tomycin and Kanamycin was used. For this process MRS agar inoculated with LAB and disc were placed. After the incubation period (24 hours/ 37°c) and inhibition zones were observed to determine the antibiotic resistance of isolates. Antagonistic activity was carried out by the method of (Bolanle *et al.,* 2015).

The agar overlay method was employed to determine the ability of the viable lactic acid bacteria strains to inhibit the growth of the indicator pathogens, *E.coli* and *Staphylococcus aureus*. A loop full of LAB in MRS broth was inoculated on MRS agar plate as a thick line of about 2mm and about 30mm long at a good away from the edge of the plates and incubated under microaerophillic condition at 37°C for 24 hours. After incubation, the MRS agar plates were overlaid with approximately 0.2ml × 10^{-7} CFU /ml of an overnight broth culture of the test pathogens inoculated in 10ml of Muller Hinton soft agar (with 0.7% agar-agar). The overlay was allowed to set and incubated at 37°C under aerobic condition. The plates were then examined for zone of inhibition around the line of the LAB and the clear zones were measured.

RESULTS AND DISCUSSION

A total of 56 LAB isolates were identified from various freshly drawn raw milk samples collected from surrounding of Tirupur and Erode district (Table 1).

All the fifty six isolates were gram positive, nonmotile in hanging drop method, fifty isolates were catalase negative and only six showed positive to catalase test were not a LAB. The cell morphology of fifty six isolates was evaluated through grams reaction microscopic observation and majority of 36 were found to be rods and the remaining of 20 isolates were cocci shaped. Among the isolates 42 were able to produce CO_2 from glucose fermentation. This result showed that they were heterofermentative and remaining of 14 isolates were homofermentative. Homofermentative LAB utilize glucose via EMP pathway and heterofementors utilize via HMP (hexose monophosphate pathway) described by Rattanachaikunsopon and Phumkhachorn (2010). All the isolates grew at 37°C but only 21 isolates were grew at 15°C;in 45°C and 55°C, only ten isolates were showed limited growth. In 3.5 % Na Cl concentration all the isolates grew well and only 27 showed growth at 6.5% Na Cl concentration. 21 isolates showed limited growth at 18% Na Cl concentration.

Based on the morphological, physiological and biochemical test chemometric result hierarchical cluster analysis (AHC) for finding homogenous group of isolates and graphics were performed using XLStat software version 2018.5, Addinsoft. Dendrogram showing the similarity relationship among the 56 isolates of LAB obtained from various raw milk. Similarities were calculated by the simple matching coefficient and grouping was performed by Agglomeration hierarchical cluster (AHC) analysis using Un-weighted pair group average linkage analysis. A total of 56 isolates characterized using set of 17 phenotypic test. An abridged dendrogram depicting the similarity relationship among the isolates were divided into six groups shown in figure 2. While the phenotypic characterization of the main clusters were summarized in linkage analysis figure 1.

Results showed that all the examined strains did not exhibited β haemolysis and most of the strains were γ haemolytic (non-pathogenic), while seven exhibited α haemolysis. Out of seven, five isolates were catalase positive there were not a LAB avoided of further work.Some of Enterococcus showed α haemolysis and as well as β haemolysis summarized by W.Liu *et al* (2014). According to the European Food Safety Authority (2012) antibiotics of Tetracyclin, Streptomycin and Kanamycin were used for antibiotic suscebtibility testing of the isolated LAB.

Table 1. Origin and designation of the isolates of Lactic acid bacteria

S. No	Sample	Region	No. of samples	No. of isolates	Designation of Strains
1	College	Tirupur, Erode	2	5	C2, C9, C16, C7, C19
2	Sindhu	Tirupur, Erode	11	11	C3, C5, C11, C13, C14,C17, C18, C20, C21, C22, C23
3	Cooli	Tirupur, Erode	2	5	C1, C6, C8, C12, C24
4	Jersey	Tirupur, Erode	1	3	C4, C10, C15
5	Buffalo	Tirupur, Erode	2	5	B25, B26, B27, B28, B29
6	Goat (Black)	Tirupur, Erode	4	8	G4,G15,G16,G21,G25,G26,G27,G1
7	Goat (white)	Tirupur, Erode	12	17	G2,G6,G13,G14,G5,G10, G7,G8,G12,G17,G9,G18, G19,G20,G22,G23,G24
8	Sheep	Tirupur, Erode	2	2	S3,S11

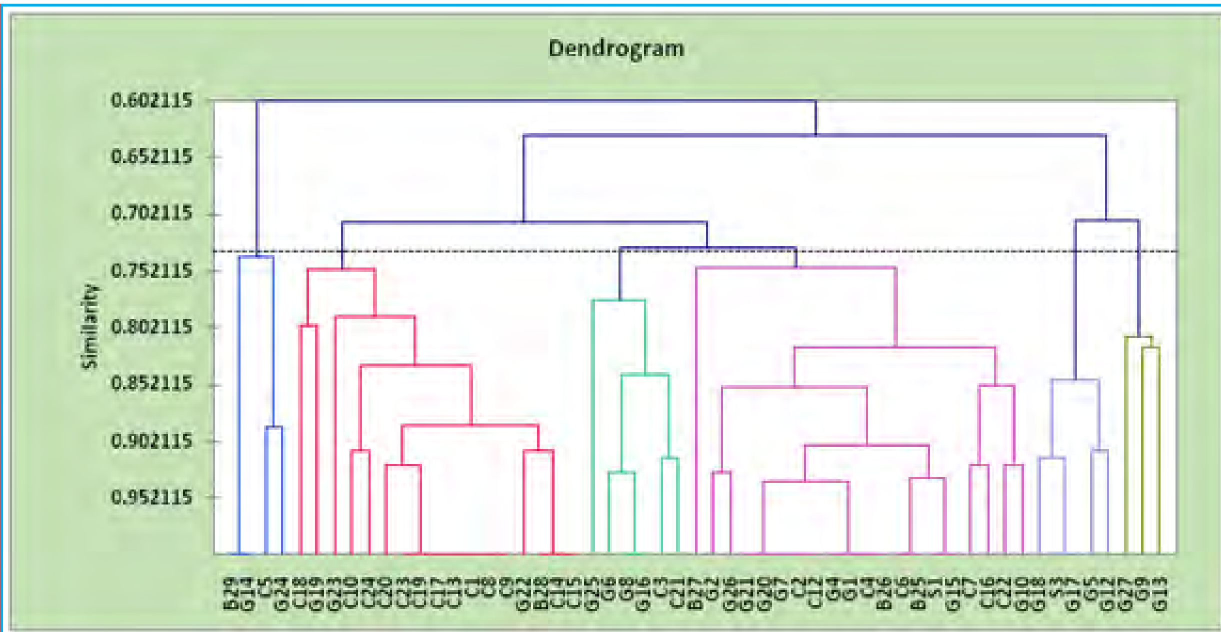


FIGURE 1. Agglomeration hierarchical clustering (AHC) of 56 isolates in raw milk

Results showed in figure 3 descripes 50% of the isolates were resistant to antibiotics. A similar observation was given by Hawaz (2014) who also noticed Ethiopia curd had *Lactobacillus* and *L. delbruekii, L.brevis* and *L. casei* were resistance to antibiotic streptomycin and gentamycin; *L. fermentum, L. lactis* and *L. rhamnosus* were resistant to streptomycin. *L. leichmanni, L. acidophillus* and *L. coagulants,* which were only sensitive to antibiotics. Furthermore Pundir et al (2013) had reported LAB isolates from food sample were found to be resistance to most of the antibiotics. Zarour et al (2012) had also reported goat and camel milk isolates were resistant to Vancomicin the strains had a poly resistance to antibiotics which is attributed to plasmid transposons in many bacterial strains. Based on the frequency of antibiotic sensitive isolates were taken for further analysis.

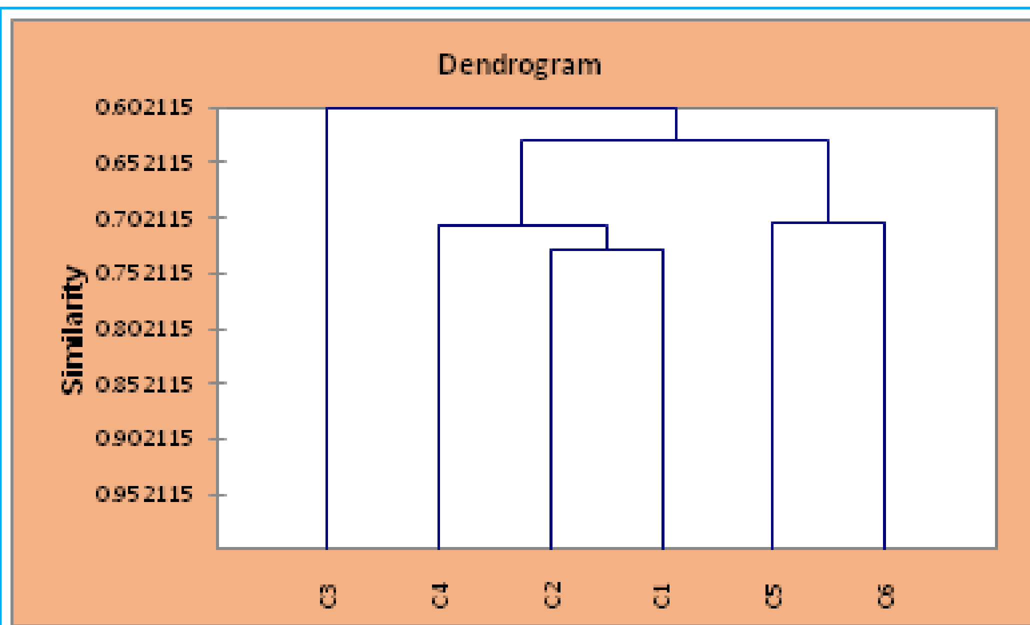


FIGURE 2. Dendrogram showed mainly six classes of 56 isolated Lactic acid bacteria in AHC

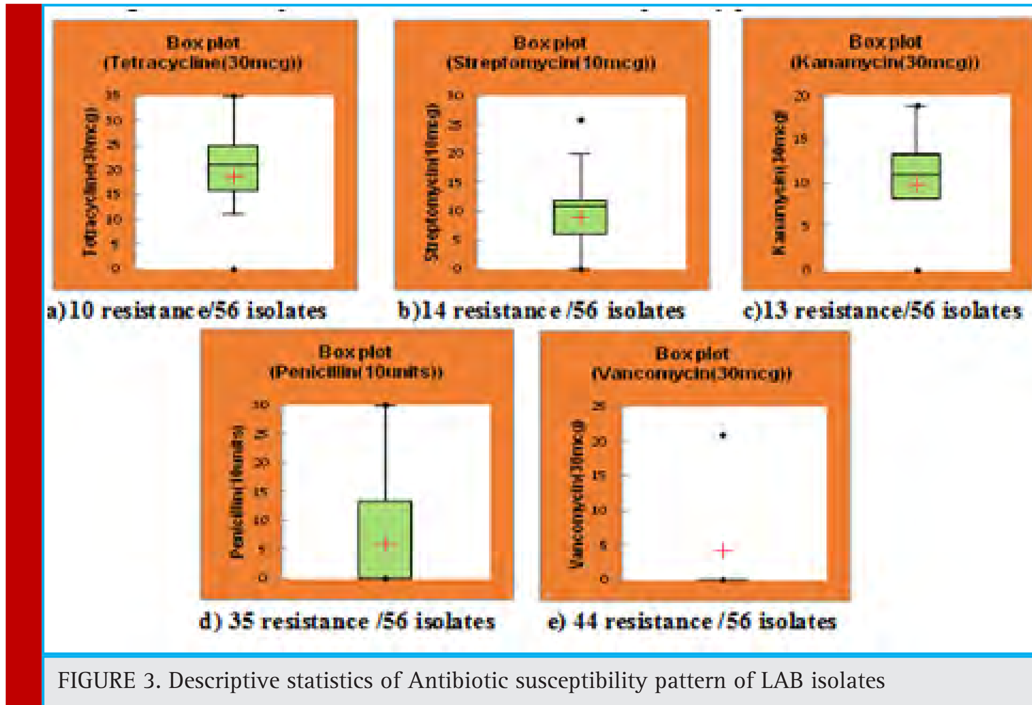


FIGURE 3. Descriptive statistics of Antibiotic susceptibility pattern of LAB isolates

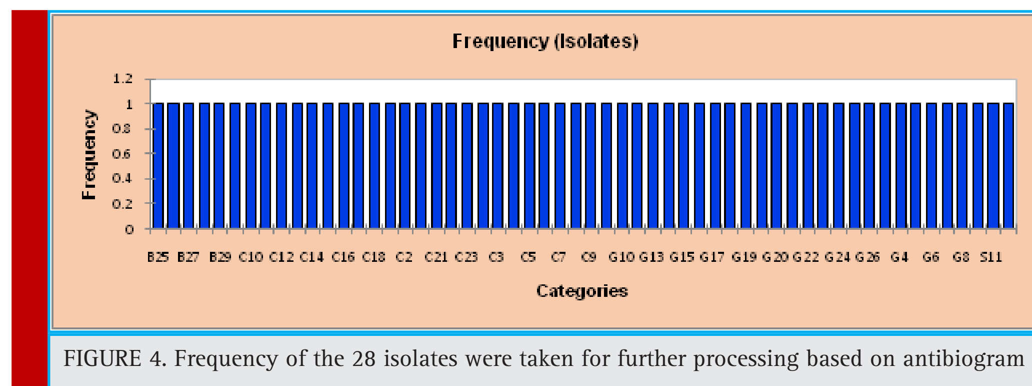


FIGURE 4. Frequency of the 28 isolates were taken for further processing based on antibiogram

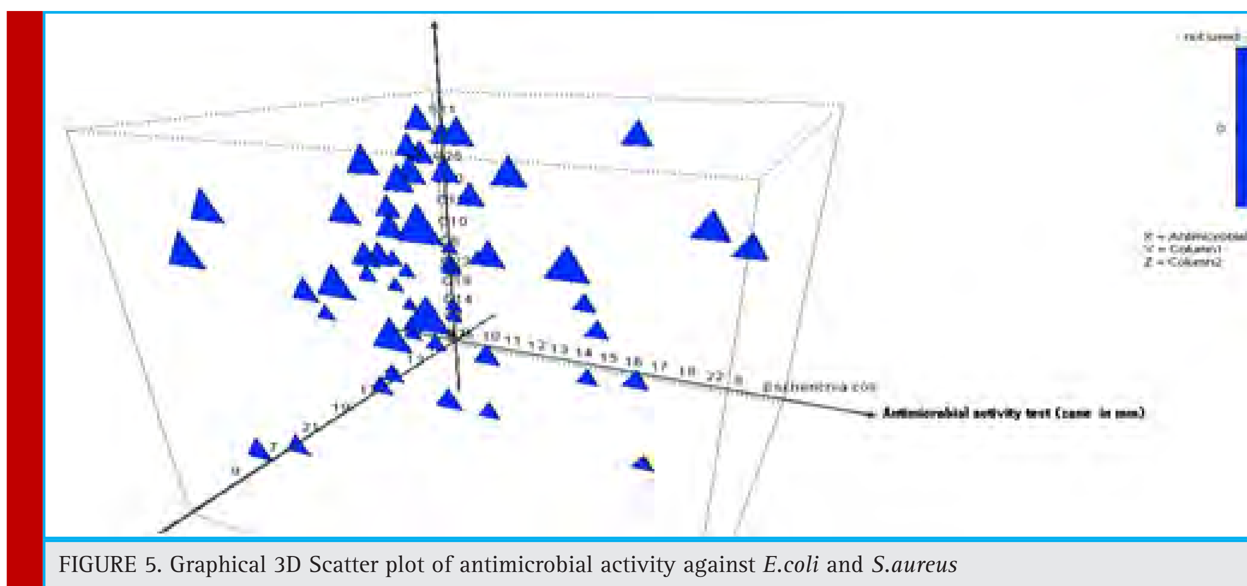


FIGURE 5. Graphical 3D Scatter plot of antimicrobial activity against *E.coli* and *S.aureus*

Table 2. Blood haemolysis, Antibiogram and antimicrobial activity of LAB isolates											
S. No	Isolates	Blood haemolysis test			Antibiotic susceptibility test (zone in mm)					Antimicrobial activity test (zone in mm)	
		α	β	γ	Tetracycline (30 mcg)	Penicillin (10 units)	Vancomycin (30 mcg)	Streptomycin (10 mcg)	Kanamycin (30 mcg)	E. coli	S. aureus
1	C1	-	-	-	R	R	R	R	R	22	19
2	C2	-	-	-	22	R	R	9	14	-	13
3	C3	-	-	-	25	10	R	12	12	-	14
4	C4	+	-	-	23	R	R	10	8	-	19
5	C5	-	-	-	R	R	R	R	R	-	13
6	C6	+	-	-	15	R	R	10	11	-	-
7	C7	-	-	-	29	R	R	12	11	11	11
8	C8	-	-	-	31	R	R	13	14	-	16
9	C9	-	-	-	23	9	R	12	12	-	14
10	C10	-	-	-	R	R	R	R	R	14	18
11	C11	-	-	-	R	R	R	R	R	-	-
12	C12	-	-	-	17	13	17	12	13	16	14
13	C13	-	-	-	22	20	R	11	12	-	13
14	C14	-	-	-	R	R	R	R	R	-	-
15	C15	-	-	-	31	R	R	12	12	-	13
16	C16	+	-	-	19	R	R	11	12	-	12
17	C17	-	-	-	22	15	R	11	11	-	12
18	C18	-	-	-	33	R	R	12	12	18	16
19	C19	-	-	-	32	10	R	16	15	14	21
20	C20	-	-	-	27	14	R	13	11	16	13
21	C21	-	-	-	22	13	R	10	11	14	18
22	C22	-	-	-	27	12	R	11	11	15	11
23	C23	-	-	-	32	21	R	26	11	-	-
24	C24	-	-	-	21	22	13	20	17	-	-
25	B25	+	-	-	18	R	19	12	13	-	22
26	B26	+	-	-	R	R	R	8	10	-	16
27	B27	+	-	-	23	R	R	13	16	-	15
28	B28	-	-	-	21	R	R	10	11	-	11
29	B29	-	-	-	31	R	21	11	12	-	8
30	G1	-	-	-	R	R	R	R	R	-	20
31	G2	-	-	-	R	R	R	R	R	-	14
32	S3	-	-	-	35	19	19	13	14	13	17
33	G4	-	-	-	22	R	R	9	11	-	11
34	G5	-	-	-	34	14	20	15	18	10	9
35	G6	-	+	-	21	R	R	10	8	-	12

36	G7	-	-	-	21	R	R	15	16	-	-
37	G8	-	-	-	22	R	R	10	10	11	19
38	G9	-	-	-	17	16	16	13	14	16	-
39	G10	-	-	-	16	R	R	R	R	-	15
40	S11	-	-	-	24	R	R	R	17	-	11
41	G12	+	-	-	30	14	16	11	12	-	16
42	G13	-	-	-	21	30	20	14	18	14	8
43	G14	-	-	-	30	17	R	13	13	13	15
44	G15	-	-	-	16	R	19	11	13	8	10
45	G16	-	-	-	22	R	R	11	11	-	14
46	G17	-	-	-	18	15	20	13	17	15	8
47	G18	-	-	-	14	R	R	11	12	12	7
48	G19	-	-	-	25	14	R	R	19	10	-
49	G20	-	-	-	17	R	R	12	14	17	20
50	G21	-	-	-	16	R	R	R	R	-	17
51	G22	-	-	-	24	R	20	R	R	-	9
52	G23	-	-	-	R	R	R	R	R	-	-
53	G24	-	-	-	21	R	R	11	10	-	13
54	G25	-	-	-	R	R	R	R	R	-	12
55	G26	+	-	-	11	10	R	10	11	14	22

In this study, the antimicrobial activity showed 46 isolates produced maximum inhibition zone against bacterial pathogen Staphylococcus sp., comparatively less isolates of 22 produced zone against *E.coli* sp. Showed in figure 4. The present work contrary with Hawaz (2014) had reported Lactobacillus strains showed antibacterial effect against the pathogenic *E.coli, Staphylococcus* sp., and *Salmonella thyphimurium*. This may vary from type of strain and class of bacteriocin produced by LAB.

CONCLUSION

The present study concluded that freshly drawn animal milk had rich source of lactic acid bacteria and predominant 64% *Lactobacillus sp.* and 34% of isolates were coccus that tentatively had *Enterococcus, Leuconostoc, Streptococcus, Lactococcus and Pediococcus* which is in conformity with earlier work. As raw milk is used more in Tamil Nadu being consumed frequently and occasionally, consumers received significant amount of LAB. Potentialities of probiotic characteristics are being done in further studies. Indeed, a natural preservation of characterized LAB in under investigation. Further work is required on species level identification of isolates and bioactive compounds produced by LAB.

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Histopathological and ultrastructural changes in the gill and liver of fresh water fish *Channa punctatus* exposed to sodium arsenite

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ABSTRACT

Arsenic, is one of the most important and concerned global environmental toxicants. Correlations have been found between chronic arsenic poisoning and many severe health effects including cancers, hypertension and ischemic heart disease etc. However, the proper understanding of the role of arsenic in the cause of these diseases is still limited. In this work, we studied the toxicity effect of sodium arsenite in the gill and liver tissues of fresh water fish *Channa punctatus* and for the first time observed the histopathological as well as surface ultrastructural changes on it. The liver and gill tissues of *Channa punctatus* were exposed to sub-lethal (12 ppm: parts per million) concentration of sodium arsenite (NaAsO2) for 96 hours. The histopathological effects of sodium arsenite on the liver and gill tissues were studied by light microscopy. The surface ultrastructural changes on the same tissues were investigated by scanning electron microscopy (SEM). The results were compared with the normal structure of liver and the gill tissue of a control group of *Channa punctatus*. Gill tissues exposed to arsenic showed hyperplasia, desquamation, and necrosis of epithelium, epithelial lifting, oedema, lamellar fusion, collapsed secondary lamellae, curling of secondary lamellae and aneurism in the secondary lamellae. Hepatic lesions in the form of cloudy swelling of hepatocytes, congestion, vacoular degeneration, karyolysis, dilation of sinusoids and nuclear hypertrophy were observed in the liver tissue of the exposed group. Thus it has been shown that sodium arsenite can produce significant damage in the ultrastructure of liver and gill tissues. Also the histological and ultrastructural changes on the liver and the gill tissue indicate that arsenic is biologically reactive and gives rise to acute poisoning.

KEY WORDS: *CHANNA PUNCTATUS*, GILL, HISTOPATHOLOGY, LIVER, SODIUM ARSENITE

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INTRODUCTION

Contamination of water by arsenic compounds and its toxicological effect on aquatic organism is a major worldwide problem. Geogenic processes and anthropogenic disturbances are the two main causes of dispersal of arsenic in aquatic environment (Bears et. al., 2006; Gonazalez et. al., 2006). Several countries including Argentina, Bangladesh, Chile, China, India, Japan, Mexico, Mongolia, Nepal, Poland, Taiwan, Vietnam, and some part of United States have been reported with high concentration of arsenic in groundwater (Anowar et. al., 2002; Mitra et. al., 2002; Smith et. al., 2001; Chowdhury et. al., 2000). A correlation has been found between chronic arsenic poisoning and many health effects including cancers, melanosis, hyperkeratosis, restrictive lung disease, peripheral vascular disease, gangrene in leg, skin, lung, bladder, liver, diabetes mellitus, hypertension and ischemic heart disease (Anawar et. al., 2002). It is evident that arsenic exposure has multiple effects at the molecular level for instance liver chromosomal DNA fragmentation, expression of certain proteins, differential expression of genes involved in cell cycle regulation, signal transduction, stress response, apoptosis, cytokine production, growth-factor and hormone-receptor production (Hossain et. al., 2003; Tabellini et. al., 2005; Ahmed et. al., 2008; Sangeeta et. al., 2012 Paruruckumani et al., 2015).

Both in laboratory and field studies histopathological investigations have been long recognised as reliable biomarkers of stress in fish and in the evaluation of the health of fish exposed to contaminants. The gills, liver and kidney are the common primary target organs for many chemicals primarily because of their vital role within the body (Chowdhury et. al., 2000; Hossain et. al., 2000, Paruruckumani et al., 2015).

In this work, we studied the toxicity effect of sodium arsenite in the gill and liver tissues of fresh water fish *Channa punctatus* and for the first time observed the histopathological as well as surface ultrastructural changes on it. We also estimated a critical value of concentration of sodium arsenite above which fishes are likely to be killed. A commonly useful measure of toxicity LC50 is used for this purpose. The goal of this study was, firstly, to observe any histological changes, arsenic could bring to the vital organs of living animal and secondly, to substantiate the role of arsenic as a toxic environmental agent which can cause many severe health effects.

MATERIALS AND METHODS

For the present study healthy and disease free fishes *Channa punctatus* (weight 22-50 gm) were collected from local markets in Guwahati. After disinfection with a dip of 2% potassium permanganate ($KMnO_4$) solution the fishes were acclimatised in aquaria for two weeks before initiation of experiment. The water provided in the aquaria was from the tap water in the laboratory and was changed on the following day. The fishes were fed everyday with fish food available in the market. Proper aeration was done during these periods. Sodium Arsenite ($NaAsO_2$), molecular weight-129.91 Merck, India (Ltd.) was procured for performing the experiment. A stock solution was prepared with water from which the test concentration was prepared by dilution. The control group of fishes were kept in similar conditions without adding sodium arsenite. Fishes were exposed to 5 different concentration of Sodium Arsenite of 5, 15, 25, 35 and 45 ppm. The toxicity bioassay was performed in semi-static system in triplicate with 10 specimens exposed for each concentration in each set in accordance with the standard methods of acute toxicity bioassay procedures (APHA, 2005).

Fishes were transferred to each aquarium and exposed to five different concentrations such as 5, 15, 25, 35 and 45 ppm of sodium arsenite. In all cases, control groups of fishes were maintained. Each experimental trial was carried out for a period of 96 hours. The mortality rate of the fish was recorded at logarithmic time intervals that is, after 6, 12, 24, 48, 72 and 96 hours of exposure. The test media was renewed daily during the experimental period. The data obtained in course of the investigation were analysed statistically to see whether there is any influence of different treatment concentrations on the mortality of the fish. Fishes were exposed to sub lethal concentration i.e. 12ppm of sodium arsenite along with a control group for 96 hours. At the end of the exposure period, fishes were randomly selected for histopathological examinations. Gill, liver, tissues were isolated from normal and experimental fish. Physiological saline solution (0.75% NaCl) was used to rinse and clean the tissue. They were fixed in aqueous Bouins solution for 24 hours, processed through graded series of alcohols, cleared in xylene and embedded in paraffin wax. Sections were cut at 4 micron thickness and stained with Hematoxylin and eosin stain. Histopathological lesions were examined and photographed with the help of computer attached Bright Field Microscope (Leica DM 3000).

Gills and liver tissues of both the control and treated groups were rapidly removed and processed routinely for scanning electron microscopic studies. Gills and liver tissues were cut into small pieces of 1 mm thickness and fixed in 2.5 % glutaraldehyde prepared in cacodylate (sodium phosphate) buffer adjusted to pH 7.4 for 24 hours and afterward washed in phosphate buffer for 15 min. After dehydration in ascending series of acetone, samples were immersed in Tetra Methyl Silane for 10 minutes at 4 degree centrigrate. Then they were brought to room temperature to dry. The specimens were mounted on Aluminium Stubs coated with gold and observed

through scanning electron microscope in Sophisticated Analytical Instrument Facility (SAIF), North-Eastern Hill University (NEHU), Shillong – 793022.

RESULTS AND DISCUSSION

The mortality rate of *Channa punctatus* to different concentration of sodium arsenite can be seen in Figure 1. In the present study, it was observed that 45 ppm sodium arsenite in water induced death of all the exposed fishes within 96 hours. The 96 hours LC50 of sodium arsenite for *Channa punctatus* was found to be 25 ppm. Fishes treated with a concentration of 5, 10 and 12 ppm survived for more than 90 days with zero mortality rates. The sub lethal concentration of sodium arsenite for the exposed group of fish was 12 ppm. The control group of fish were in good condition without any morphological changes. But the sodium arsenite treated fish showed rapid movement of fins and operculum. They produced a lot of slime around their body. Their overall activities decreased with time.

In the liver tissue of control *channa punctatus*, there was normal structure and systematic arrangement of hepatocytes. Hepatic cells were roundish, polygonal containing clear spherical nucleus which can be seen in the Figure 2. The normal histological arrangement was

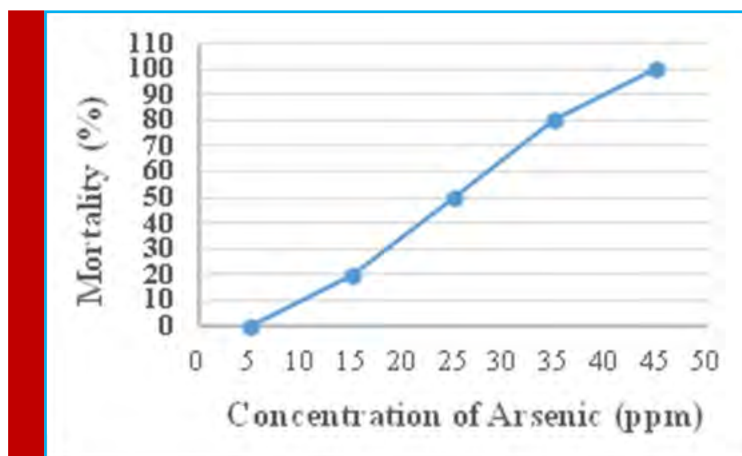

FIGURE 1. Graphical representation of 96 hours LC50 of Sodium arsenite treated *Channa Punctatus*. It shows mortality rate of *Channa Punctatus* to different concentration of sodium arsenite, (ppm: parts per million).

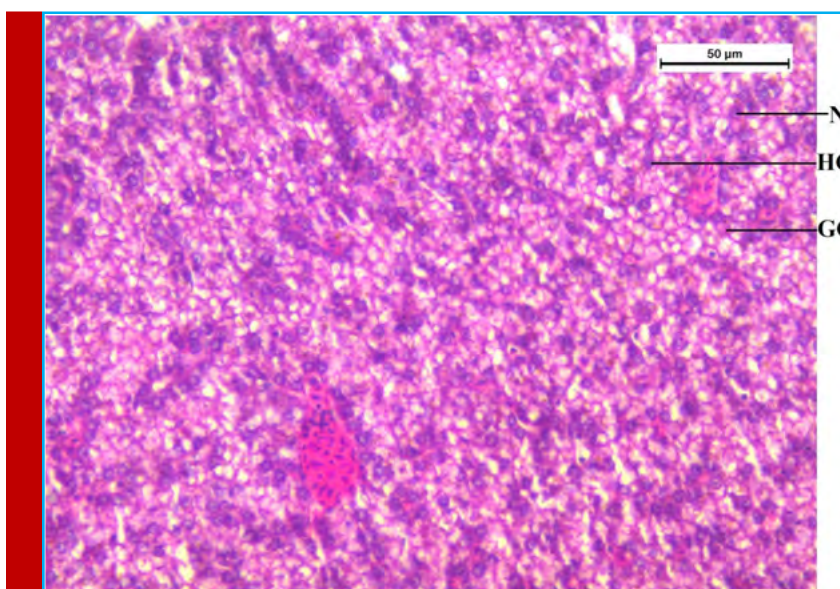

FIGURE 2 Optical Micrograph of liver tissue of control group of *Channa punctatus*. (N-Nucleus, HC-Hepatic cell, GC-Granular cytoplasm).

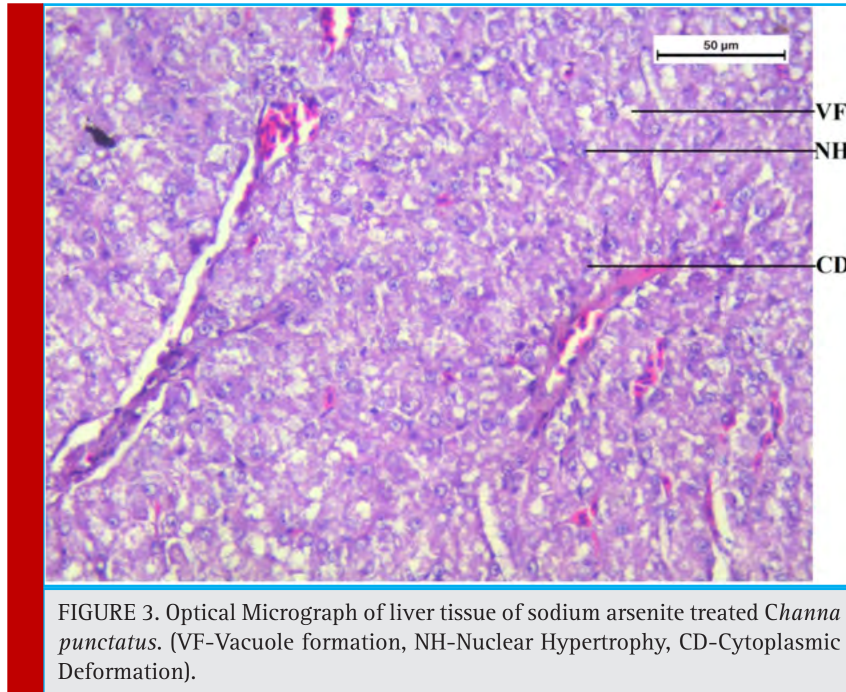

FIGURE 3. Optical Micrograph of liver tissue of sodium arsenite treated C*hanna punctatus.* (VF-Vacuole formation, NH-Nuclear Hypertrophy, CD-Cytoplasmic Deformation).

not found in the liver tissue of sodium arsenite treated *channa punctatus.* A micrograph of liver tissue of sodium arsenite treated *channa punctatus* is shown in the Figure 3. The micrograph shows a lot of rupture of blood vessels, necrotic tissue with marked loss of hepatocytes and extensive area of vacuolation in the liver tissue. Figure 3 also reveals large lipid droplets and abundant glycogen in most of the area of hepatocytes of liver tissue.

A Scanning Electron Micrograph of liver tissue in control *channa punctatus* is shown in the Figure 4 which represents normal ultrastructural morphology of hepatocytes. Serous membranes with some connective tissue are seen in the surface of the liver tissue. Hepatic cells are seen with clear spherical nucleus. Liver is the primary organ for detoxification of foreign compounds (Gernhofer et al., 2011) and one of the most affected

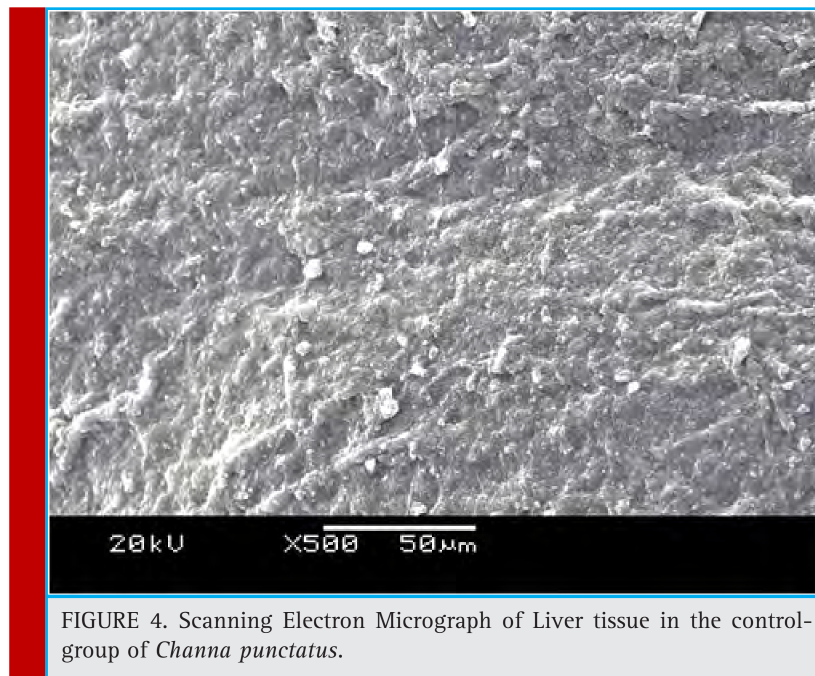

FIGURE 4. Scanning Electron Micrograph of Liver tissue in the control-group of *Channa punctatus.*

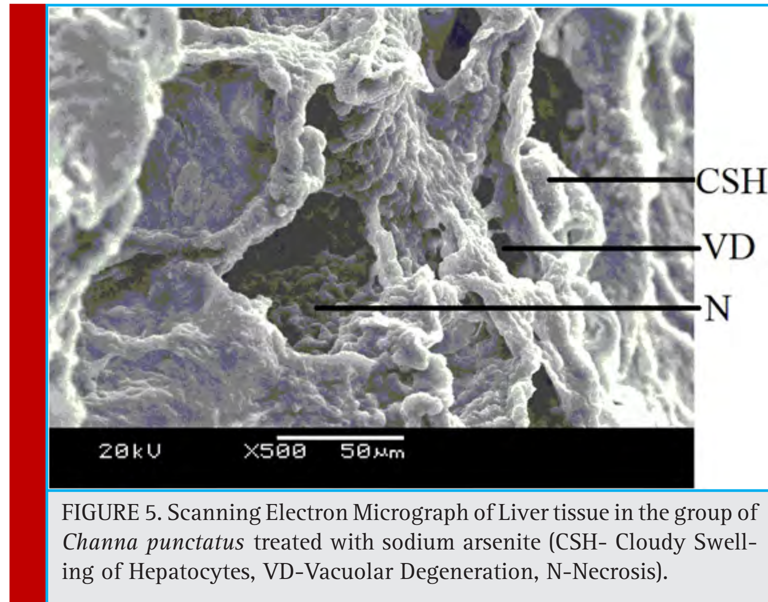

FIGURE 5. Scanning Electron Micrograph of Liver tissue in the group of *Channa punctatus* treated with sodium arsenite (CSH- Cloudy Swelling of Hepatocytes, VD-Vacuolar Degeneration, N-Necrosis).

organs by contaminants in water (Camargo, Martinez, 2007). In our study it has been found that sodium arsenite caused several damages in the liver tissue which includes destruction of normal arrangement of the cells, vacuolar degeneration of cytoplasm, necrosis and cloudy swelling of hepatocytes. These changes are represented in the Figure 5. In earlier studies (Ahmed et al., 2008; Sangeeta et al., 2012) on sodium arsenite treated *Channa punctatus* showed concentration dependent reduced cell viability and chromosomal DNA fragmentation of liver cells. Finding of Ahmed et al., 2008, revealed that lower concentration of sodium arsenite induced apoptotic death of cells while higher concentration induced necrotic cell death.

In *Channa punctatus* there are four pairs of semicircular gill arches. Each gill arch has a row of microscopic primary gill lamellae on which secondary gill lamellae are arranged bilaterally. In the control group normal structure of gill lamellae were observed (Figure 6). The histology of the treated sub lethal exposure revealed loss of structural integrity of lamellae. It also shows destruction of cartilaginous gill bar, degenerated primary and secondary gill lamellae, lamellar fusion and capillary lumen and that can be clearly seen from Figure 7. Anal-

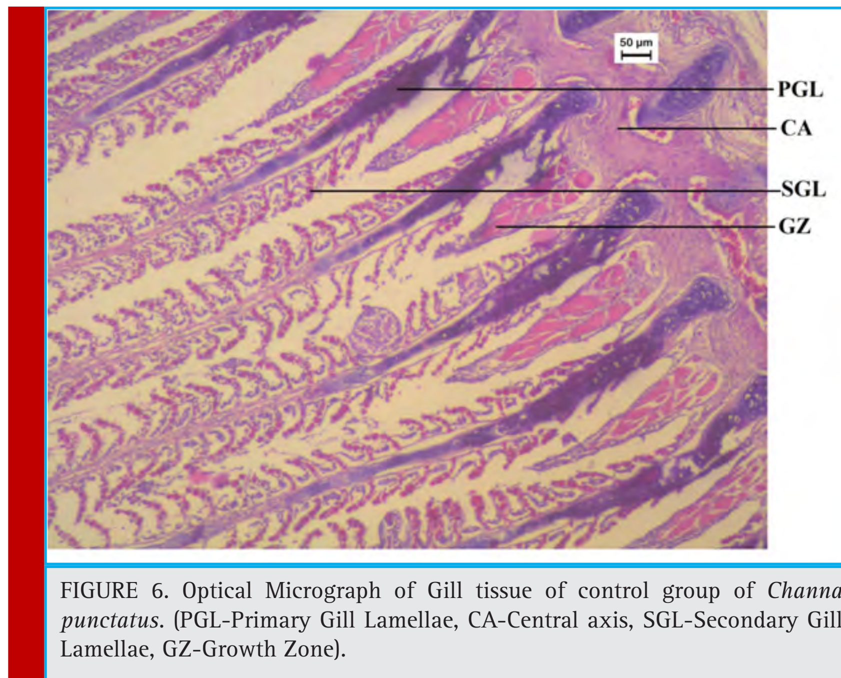

FIGURE 6. Optical Micrograph of Gill tissue of control group of *Channa punctatus*. (PGL-Primary Gill Lamellae, CA-Central axis, SGL-Secondary Gill Lamellae, GZ-Growth Zone).

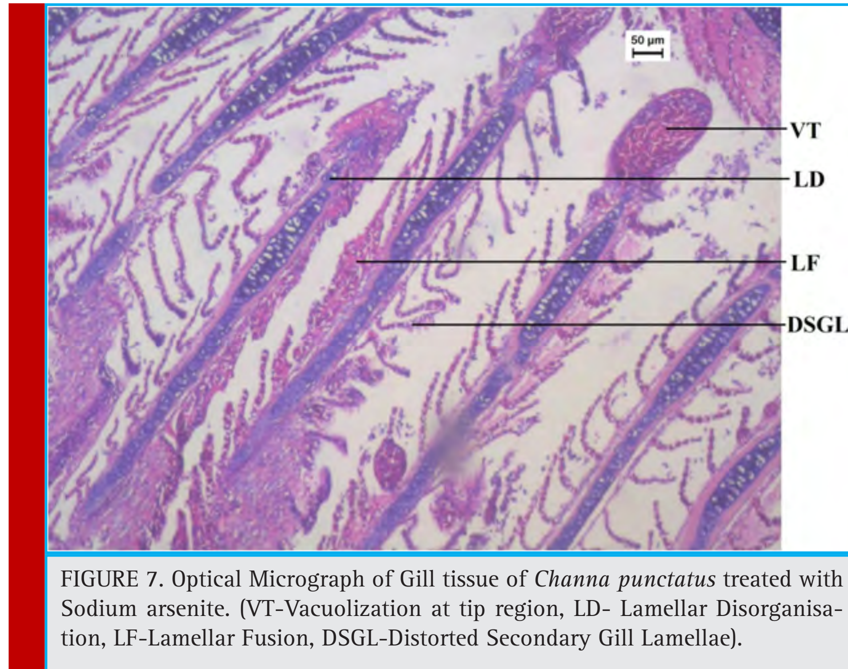

FIGURE 7. Optical Micrograph of Gill tissue of *Channa punctatus* treated with Sodium arsenite. (VT-Vacuolization at tip region, LD- Lamellar Disorganisation, LF-Lamellar Fusion, DSGL-Distorted Secondary Gill Lamellae).

ogous structural changes could be seen from the gill tissue of *Channa punctatus* exposed to arsenic trioxide (Agnihotri et al., 2010). Their findings revealed degenerative changes in cartilaginous bar and increased mucous secretion between the spaces of primary gill lamella while capillary lumen developed enlarged spaces in gills of *Channa punctatus* exposed to arsenic trioxide. The secondary gill lamellae of arsenic trioxide treated fish showed destruction of epithelial cells, vacuolization in the tip of the primary gill ray, gill hyperplasia and lamellar fusion (Agnihotri et al., 2010). Pathological lesions in the gill tissue induced by sodium arsenite were similar to cadmium induced gill tissue of *Labeo rohita* (Muthukumaravel et al., 2013). Copper induced gill tissues of *Oreochrombis mossambicus* showed marked alternations which were studied by Radhika and Krishnamoorthy (Radhika et al., 2010).

Figure 8 shows a normal architecture of gills in the control group of fish. Normal structure of primary gill lamella, secondary gill lamella and micro ridges on the normal gill epithelium were observed. In the gill tissue of sodium arsenite treated fish fusion of secondary lamella, necrosis and deformation of the gill tissue were observed and that can be seen in the Figure 9. The SEM micrograph of gill in sodium arsenite treated *Channa Punctatus* (Figure 9) also reveals swelling and curling

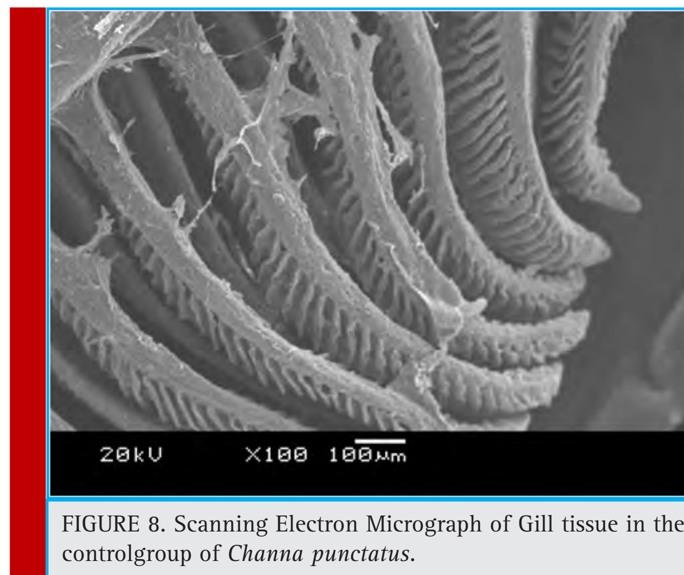

FIGURE 8. Scanning Electron Micrograph of Gill tissue in the controlgroup of *Channa punctatus*.

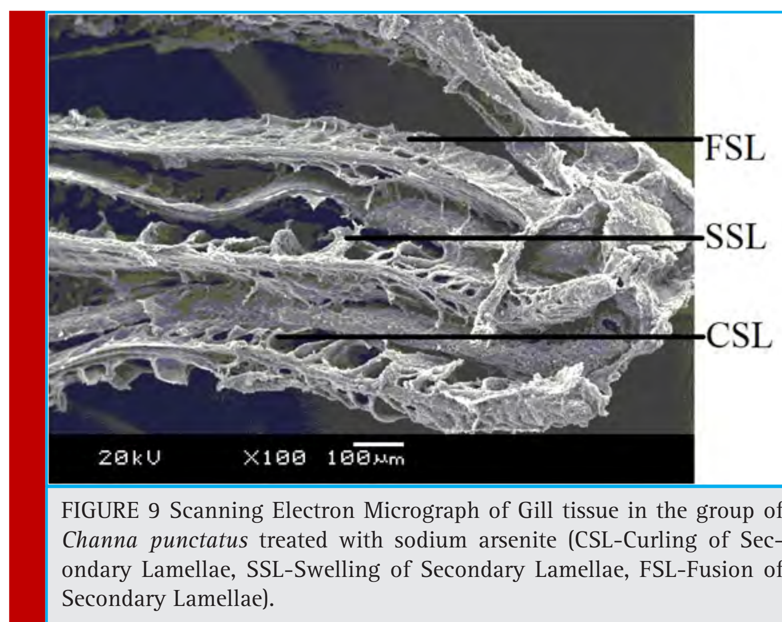

FIGURE 9 Scanning Electron Micrograph of Gill tissue in the group of *Channa punctatus* treated with sodium arsenite (CSL-Curling of Secondary Lamellae, SSL-Swelling of Secondary Lamellae, FSL-Fusion of Secondary Lamellae).

of secondary lamellae, complete fusion of secondary lamellae and surface wrinkling in numerous areas of the gill tissue. These observations are in accordance to those reported in Surface ultrastructural changes in the gill and liver tissue of Asian sea bass *Lates calcarifes* (Bloch) exposed to copper (Paruruckumani et al., 2015)

CONCLUSION

This work presents a unique evidence of arsenic toxicity in fishes and how its sub lethal concentration causes ultrastructural damages on gill and liver tissue. We also have seen high sensitivity and behavioural changes in the treated fish. The data obtained from the concentration dependent study of sodium arsenite to *Channa punctatus* can be used to set a standard for human exposure to arsenic. Further studies on the nature of arsenic induced damages observed on the cellular structure of the concerned tissue could provide some insight into the mechanism of arsenic poisoning on human being.

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Formulation of detection bioconjugates of gold coated iron oxide nanoparticles for aflatoxin M1 in milk

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ABSTRACT

Mycotoxins particularly aflatoxins are gaining increasing importance due to their deleterious effects on human and animal health and also due to ubiquitous presence of aflatoxigenic fungi in all the agricultural products. Corn, groundnuts and other plants were infected by *Aspergillus flavus & Aspergillus parasiticus* and secrete the mycotoxins. Aflatoxin B1 transmitted to cow by feeding these infected plants and aflatoxin B1 transformed into its hydroxylated product such as aflatoxin M1 and M2 and such aflatoxin secreted in cow milk. In the present study, we explored the nanobiotechnology approach to prepare detection bioconjugates of gold coated iron oxide nanoparticle for the detection of aflatoxin M1 in milk. Gold coated iron oxide nanoparticles (Au-Fe3O4 NPs) ranging between 10-20 nm were synthesized by co-precipitation method and functionalized by EDC-NHS then labeled with FITC-labeled streptavidin (FITC-STV). Biotinylated aflatoxin M1 (biAFM1) specific to monoclonal anti-aflatoxin M1 antibodies (mAFM1) were prepared, separately. The fluorophore FITC-STV-Au-Fe_3O_4 NPs and bimAFM1 antibodies were allowed to interact to obtain Au-Fe_3O_4 NPs-FITC-STV-bimAFM1 antibody and AFM1 bioconjugates. The bioconjugates were characterized by Transmission Electron Microscope (TEM), Scanning Electron Microscope with Energy Dispersive X-ray (SEM-EDAX), Fourier Transform Infrared (FTIR), Particle Size Analyzer with zeta potential (PSA) and Fluorescence Microscopy. The bioconjugation formation was confirmed by pinkish red color in bright flied microscopy same field observed by fluorescence microscopy shows the green fluorescence which confirm the aggregation between Au-Fe3O4 NPs-FITC labeled STV-bimAFM1 antibody and AFM1.

KEY WORDS: AFLATOXIN M1, BIOCONJUGATES, GOLD COATED IRON NANOPARTICLE, MONOCLONAL ANTIBODY AFM1

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INTRODUCTION

From the last decade iron oxide nanoparticles (Fe_3O_4 NPs) and their combinations with gold (core/shell) has become more attractive because of their vast applications in different fields such as biosensor, medical field and drug delivery etc. The surface binding of various chemical and biological molecules onto gold particle was due to thiol chemistry of gold surface for attachment of functionalized compounds (Kouassi *et al*., 2007). Gold coated iron oxide nanoparticle (Au-Fe_3O_4 NPs) were synthesized by reduction of metal with sodium borohydride through sonication method (Baniukevic *et al.,* 2013). Using hydroxylamine as a reducing agent gold (shell) coated iron oxide (core) nanoparticle also synthesized by iterative reduction method and it analyze by transmission electron microscope (TEM) and superconducting quantum interference device (SQUID) magnetometer reported (Lyon *et al.,* 2004). The measurement of fluorescent and optical properties of Au-Fe_3O_4 NPs has been reported by (Baniukevic *et al*., 2013 Carrasco et al., 2018).

Corn, groundnuts and other plants were infected by *Aspergillus flavus* and *Aspergillus parasiticus* and secrete the mycotoxins which is responsible for the food born disease. Corn, groundnuts and other plants were infected by *Aspergillus flavus* and *Aspergillus parasiticus* and secrete the mycotoxins. Aflatoxin B1 transmitted to cow by feeding these infected plants and aflatoxin B1 transformed into its hydroxylated product such as aflatoxin M1 and M2 and such aflatoxin secreted in cow milk which quite stable during storage, pasteurization and milk product preparation (Stroka & Anklam, (2002). The aflatoxin is high temperature resistant and is not inactivated after milk treatment processes such as pasteurization, sterilization and others. Mycotoxins particularly aflatoxins are gaining increasing importance due to their deleterious effects on human and animal health and also because of ubiquitous presence of aflatoxigenic fungi in all the agricultural products. Animals contact to mycotoxins by consumption of infected food, it may be harmful to their health as well as humans, who are consumers of the animal products such as milk (Gacem and Hadj-Khelil 2016, Ketney *et al.,* 2017).

Aflatoxin is a kind of mycotoxin that was discovered from the mass poisoning of turkeys in Britain in 1960, and has strong carcinogenicity. Aflatoxin M1 (AFM1) is hydroxylated product of aflatoxin B1. Numerous diagnostic methodology like, chromatographic method includes, TLC, HPLC and OPLC, FT-NIR and enzyme-linked immunosorbent assay (ELISA) were available for the detection of aflatoxins but it is time consuming and requiring sophisticated instruments as well as trained manpower (Espinosa *et al.,* 2011). Diagnostic immunoassay have been use with specific antigen antibody interaction for the detection of many molecules such as AFM1 because of their sensitivity, easy to handle and quantitatively measurements. In fluorescence microscopic technique antibody labeled with so many fluorescent dyes such as Fluorescein isothiocyanate (FITC), Rhodomine, Alexa fluor and other are used as an indicator which provide optical contrast for better analysis (Adarsha *et al.,* 2015).

Till now, the use of gold coated iron oxide nanoparticle was comparatively less because it require more time to synthesis, require high temperature for the synthesis of nanoparticle and very difficult to prevent aggregation without chemical modification or use of surfactant. Here in this study, we have used simple and rapid method for the synthesis of gold coated iron oxide nanoparticle at room temperature by chemical co-precipitation, reduction of sodium borohydride and sonication method. In first step, synthesis of iron oxide nanoparticle as a core by chemical co-precipitation and then it coated with gold as shell by reduction of sodium borohydride with sonication to enhanced particle monodispersity. Then prepared particles were characterized by Transmission Electron Microscope (TEM), Scanning Electron Microscope with Energy dispersive X-ray (SEM-EDAX), Fourier Transform Infrared (FTIR), Particle Size Analyzer with zeta potential (PSA) and Fluorescence Microscopy. Surface charges and further modifications with mercaptopropionic acid and EDC-NHS also characterization by the same.

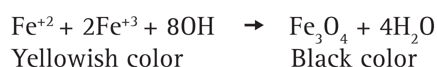
Our research effort is in direction of the detection of AFM1 with biotin-streptavidin binding approach by labeling of fluorescence dye with gold coated iron oxide nanoparticle. The bioconjugation formation between streptavidin-AFM1 and biotinylated monoclonal antibody of AFM1 was confirmed by fluorescence microscopy shows the fluorescence compounds which confirm the interaction. Mycotoxins particularly aflatoxins are gaining increasing importance due to their deleterious effects on human and animal health and also due to ubiquitous presence of aflatoxigenic fungi in all the agricultural commodities under field and storage conditions. Common established methodologies for aflatoxin detection include thin-layer chromatography (TLC), (Flores and Gonzalez 2017) and high performance liquid chromatography (HPLC) (Carrasco *et al.,* 2018).

These techniques have excellent sensitivities but they require skilled operators, extensive sample pre-treatment and expensive equipments. The present investigation was an attempt to develop basic mechanism of new nanotechnology based detection system with a minimal size, weight and real low cost and rapid detection will have significantly impact the practice of monitoring program for aflatoxin.

MATERIAL AND METHODS

All chemicals were obtained from commercial source and used as received. $FeSO_4.7H_2O$, $FeCl_3$, $HAuCl_4$, FITC-streptavidin, biotin and NHS-biotin, aflatoxin M1 (AFM1) were purchased from Sigma Aldrich (USA). Sodium hydroxide, perchloric acid, sodium borohydrate, cetyltrimetyl–ammonium bromide (CTAB), ethylene diamine tetra-acetic acid (EDTA), 3-mercaptopropionic acid, N-hydroxysuccinimide (NHS), N-Ethyl-N-(3-dimethyl-aminopropyl)-carbodimide, (EDC), streptavidin (STV), Dimethyl Sulfoxide (DMSO), phosphate buffer solution (PBS) at pH 7.4 HPLC grade pure water, were purchased from HiMedia (India). Aflatoxin M1 monoclonal antibody (mAFM1-Ab) were purchased from MyBiosource (USA). All chemicals materials were used as received.

The synthesis of Fe_3O_4 NPs were carried out by modified Massart's co-precipitation method. The ratio of iron salt (Fe(III)/Fe(II) was kept as 2:1 in an alkaline solution. Briefly, 0.64M $FeCl_3$ and 0.32M $FeSO_4.7H_2O$ were dissolved in 40ml of deionized water and the solution was stirred (TARSON SPINOT DIGITAL) until the iron salt completely dissolved. Subsequently add 40ml of 1M NaOH solution drop wise into stirred mixture and stirred it for 20minutes. The formation of Fe_3O_4 (magnetite) nanoparticle by indicating the color change from yellowish brown to black. The chemical reaction of magnetite precipitation may be written as follows (Tamer *et al.*, 2010).



The black color precipitate was separated by permanent magnet and washed with deionized water. To obtain oxidized magnetite nanoparticles, separated black precipitate was first washed with 2M perchloric acid and waited for 2-3 hours to oxidized iron salt to magnetite until the color was change from black to brown under inert condition. Then particle was centrifuged (REMI CM-12plus) at 10,000 rpm for 20 minutes. After centrifugation, discard the supernatant and washed with deionized water. Repeat he washing procedure for 2-3 times or until pH was reduced to neutral pH. The gold coating procedure was carried out in the presence of CTAB to encapsulate the synthesized Fe_3O_4 NPs by gold as a shell. Briefly, 10mg Fe_3O_4 NPs were add into 5ml 0.27M EDTA solution which prepared in 1M NaOH. The mixture was stirred in sonicator (LABMAN) at 30 amplitude for 5 minutes. Then resulting solution was centrifuge at 10,000 rpm for 10 minutes. Supernatant was discarded and pellet was washed with deionized water. Washing procedure was repeated for 3 times. Then add 7ml of 0.1M CTAB, 3ml 0.01M $HAuCl_4$ and 300µl 1M NaOH into resulting precipitation and stirred vigorously, subsequently add 150mg sodium borohydrate into stirred mixture and stirred vigorously for 3minutes. The color was change from yellow to dark red indicated the gold was coated into core (iron oxide nanoparticles (Fe_3O_4 NPs) as a shell (Tamer *et al.*, 2010).

SYNTHESIS OF GOLD COATED IRON OXIDE NANOPARTICLES WITH FITC LABELED STREPTAVIDIN AS A CARRIER MOLECULE

10mg gold coated iron oxide nanoparticles were added in 1ml of 20 mM 3-mercaptopropionic acid and sonicated for 4 hours. After sonication particles were washed with pure water. This procedure createdf the carboxyl group onto surface of gold coated iron oxide nanoparticles with EDC and NHS linked and it was used as a carrier material for streptavidin binding. For cross link of carboxylated nanoparticles to EDC-NHS, first 5mg of nanoparticle was added into 2ml EDC (0.05mg/ml) solution containing 0.2% NHS prepare in cold water then it sonicate for 5 minutes at 4°C, after sonication particles were separated out by using permanent magnet. After this process, add 1ml of 2mM streptavidin prepared in PBS (pH-7.4) in separated nanoparticle, mix it properly then incubate it for 2 hours.

Streptavidin fluorescently labelled with Fluorescein isothiocyanate (FITC) at a stock concentration of 1 mg/mL. This dye has an absorption peak at 495 nm and an emission peak of 525 nm. The resultant FITC labeled streptavidin binded gold coated iron oxide nanoparticles were separated by permanent magnet from reaction mixture, then air dried for 12 h and resuspended in water. The procedure was repeated for three times to remove impurities (Eivari and Rahdar (2013). Anti-AFM1 monoclonal antibody having sulfosuccinimidyl group was prepared separately by covalently binding primary amines of antibody (Gretch (1987). 10mg (10mM) NHS-biotin solution in 1ml DMSO was added and dissolved in it and was prepared prior to use. Then was added monoclonal AFM1 antibody with 80µg of NHS-biotin/mg ratio, mixed immediately and was incubated for 3 hours in shaking condition. After incubation period dialysis procedure of NHS-biotin binded antibody was carried out at 4°C for overnight to remove unbinded NHS-biotin molecules. Subsequently, store the biotin labeled AFM1 antibody at 2-8°C.

The procedure was started with 50µl biotinylated monoclonal AFM1 antibody and 20µl streptavidin conjugated gold coated iron oxide nanoparticles were mix together and incubated at 4 °C for 1 h in dark condition. The biotinylated antibody had bound non-covalently to streptavidin and had formed bridge for direct sensing of aflatoxin M1. After the formation of nanoparticle bio-

conjugate add 10 µl AFM1, it was incubated at 4 °C for 30 minutes in dark condition. Characterization of bio-conjgates aggregation was done by different techniques (Adarsha *et al.*, 2015).

RESULTS AND DISCUSSION

TRANSMISSION ELECTRON MICROSCOPE

The morphological characterization iron oxide nanoparticle was done by transmission electron microscopy (TEM). A small drop of formulated iron oxide nanoparticle was placed on the copper grid surface and dried it at room temperature. TEM analysis was carried out by JEOL-JEM2100 transmission electron microscope.

The shape of nanoparticles was spherical having average dimension of 10.8 ± 4.6nm. The TEM image of Fe_3O_4 NPs shows in Figure 1. The morphology and magnetic properties can be controlled by varying in pH solution, ionic strength, temperature, reaction time, type of salts and stirring speed. Eivari and Rahdar (2010) has reported that Fe_3O_4 NPs was almost spherical and their mean size was 10 nm. Our TEM result of Fe_3O_4 NPs morphology is at par to this.

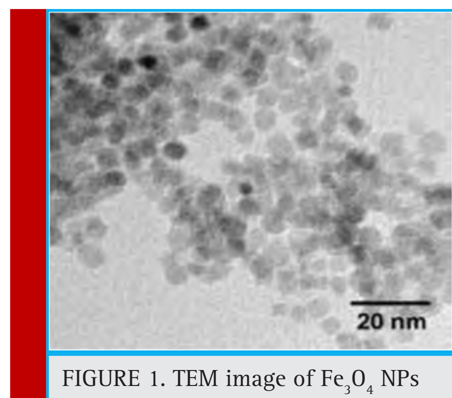


FIGURE 1. TEM image of Fe_3O_4 NPs

Figure 2 shows that the Au-Fe_3O_4 NPs was darker than Fe_3O_4 NPs. TEM images shows that the average particles size was increased from 10nm to 14nm after gold coating. The reduction of gold onto spherical surface of Fe_3O_4 NPs and it has average size of Au-Fe_3O_4 NPs was 14nm ± 3nm. Eivari and Rahdar (2010) has reported that the after coating of gold onto the Fe_3O_4 NPs it appear more darker than Fe_3O_4 NPs because of gold having more electron density then iron. Our TEM result of Au-Fe_3O_4 NPs is at par with them.

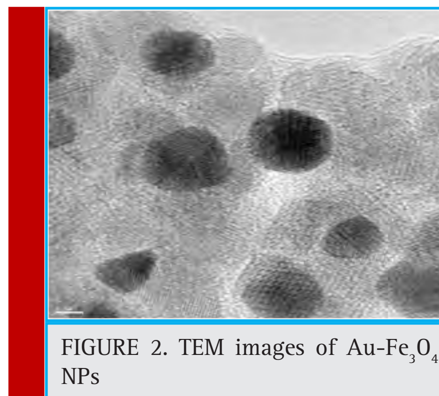


FIGURE 2. TEM images of Au-Fe_3O_4 NPs

SCANNING ELECTRON MICROSCOPE (SEM) WITH ENERGY DISPERSIVE X-RAY (EDAX)

The formulated Au-Fe_3O_4 NPs were further examine using a Zeiss EVO-18 Scanning Electron Microscope with Energy Dispersive X-Ray facility (SEM with EDAX), operating at 20 kV in vacuum and at 20KX magnification.

Confirmation of the morphology of the formulated Fe_3O_4 NPs and Au-Fe_3O_4 NPs analyze by SEM with EDAX which show in Figure 3.

After the synthesis of Fe_3O_4 NPs it was coated by gold with reduction of sodium borohydride in the sonication

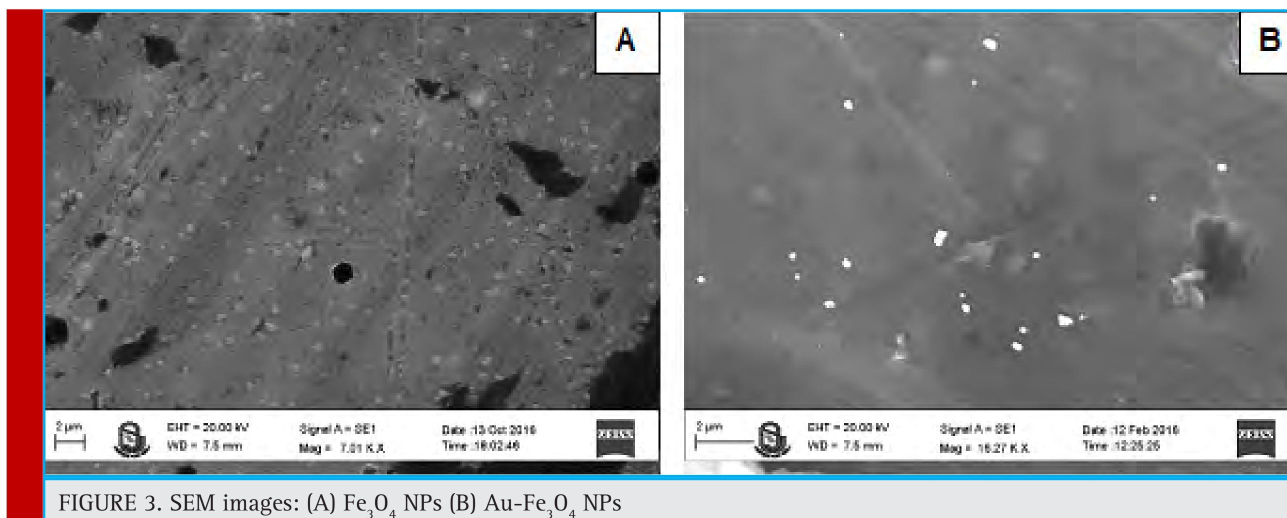


FIGURE 3. SEM images: (A) Fe_3O_4 NPs (B) Au-Fe_3O_4 NPs

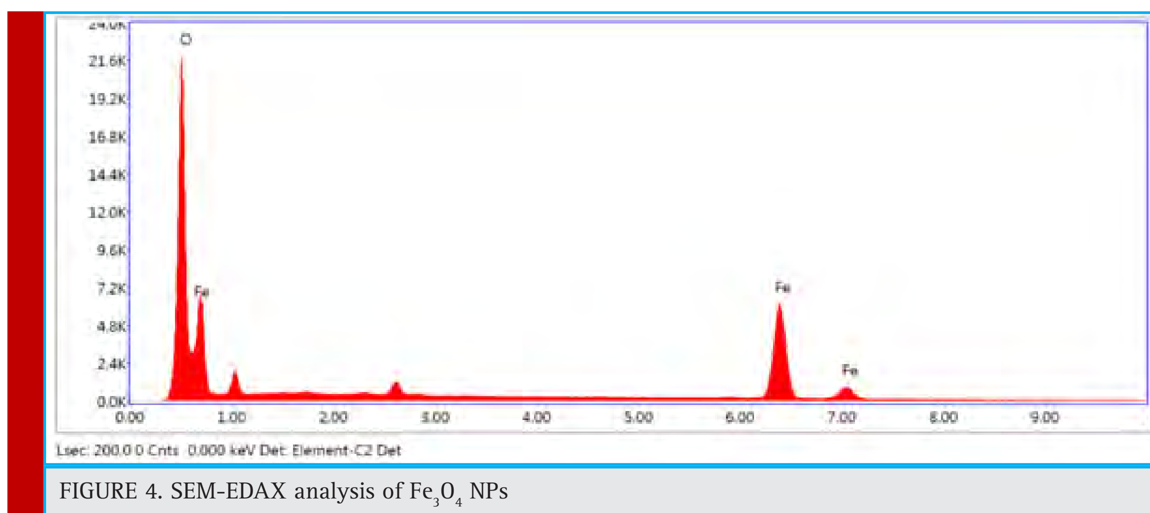


FIGURE 4. SEM-EDAX analysis of Fe_3O_4 NPs

chamber which shows in Figure 4. The difference in the size of Fe_3O_4 NPs shows that the coating of gold onto Fe_3O_4 NPs was may be successful.

The analysis of Au-Fe_3O_4 NPs by EDAX was done to investigate the presence of Au and Fe in the synthesized nanoparticle. EDAX spectrum of the Au-Fe_3O_4 NPs was shown in Figure 5, which confirms the existence of Au and Fe in the synthesized nanoparticle. Hoskins *et al.,* (2012) has reported the presence of Au-Fe_3O_4 NPs by EDAX analysis. Our EDAX analysis result of Au-Fe_3O_4 NPs is affirmation to this.

PARTICLE SIZE ANALYZER AND ZETA POTENTIAL

In the formulation of Fe_3O_4 NPs experiments, known volumes (normally 500 µL) of Fe_3O_4 NPs suspension were taken for particle size analyzed by a Malvern Mastersizer 2000. The particle size analyzer utilizes laser technology based on the Mie light-scattering theories. Fe_3O_4 NPs was characterized to examine the particle mean size and understand the properties under different physiological conditions and also determine the surface charges of nanoparticle.

The average size of Fe_3O_4 NPs was 52.04nm ± 5nm shown in Figure 6. The Zeta Potential of synthesized Fe_3O_4 NPs was -31.0 mv and polarity was negative, so it has good particle stability reflated by this result. Our result is affirmation to study of Behera *et al.,* (2012).

VIBRATING SAMPLE MAGNETOMETER

Iron oxide (magnetic) nanoparticle may give different magnetic properties depending on condition of formulation Gupta and Gupta (2005). At room temperature iron oxide nanoparticle has superpara-magnetic property where at 60 emu/g reported for saturation. The vibrating sample magnetometer measure the iron oxide nanoparticle and gold coated iron oxide nanoparticle characteristic at 300K.

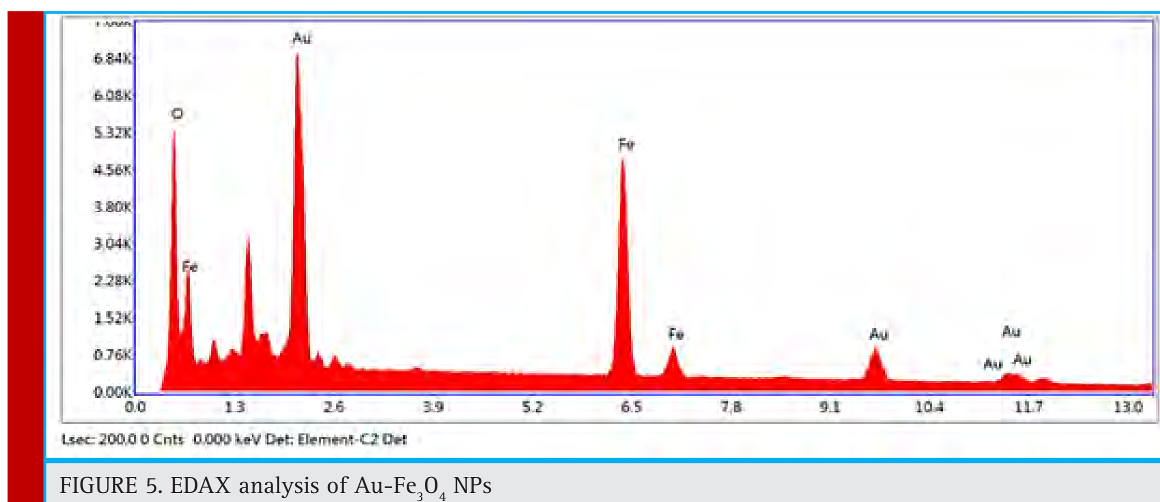


FIGURE 5. EDAX analysis of Au-Fe_3O_4 NPs

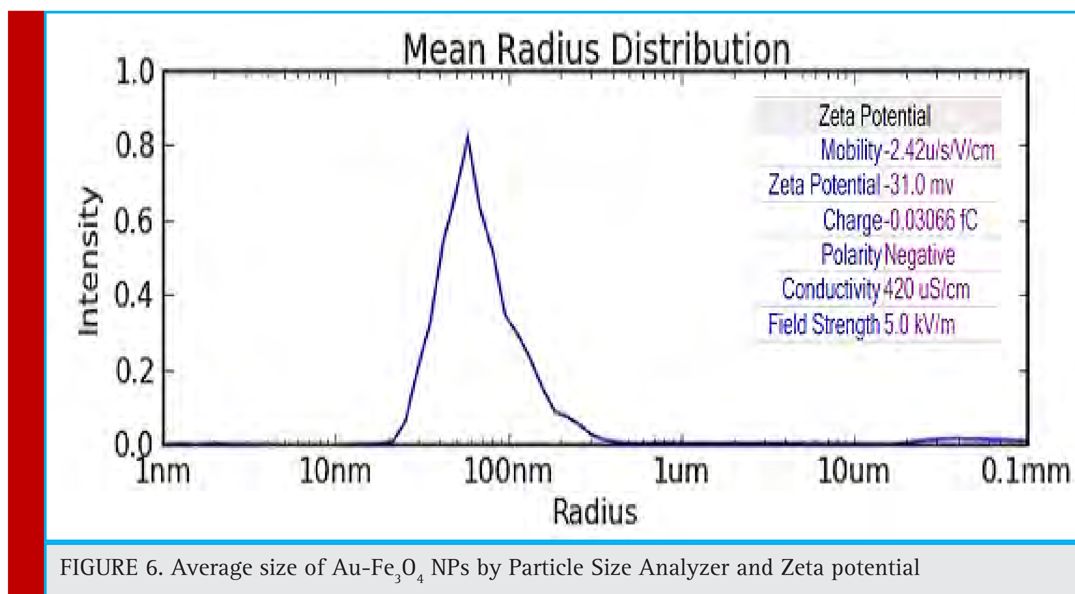


FIGURE 6. Average size of Au-Fe_3O_4 NPs by Particle Size Analyzer and Zeta potential

Figure 7 shows hysteresis loop of uncoated & gold coated iron oxide nanoparticle. The superpara-magnetism was observed in both synthesized nanoparticles. The magnetism form saturation to superpara-magnetism curve the value of iron oxide nanoparticle and gold coated iron oxide nanoparticle was 30 and 4.5 emu/g respectively at 300K. Reduction in magnetism was indicated that the magnetism was indicated that the formation of gold shell into iron oxide nanoparticle core.

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPIC ANALYSIS

The carboxylic group attached on the surface of Au-Fe_3O_4 NPs by treatment of ethanolic solution having 3-mercaptopropionic acid. The activation of carboxylic group for the bonding between amide and carboxylic group, the activation of carboxylic group was catalyzed by carbodimide (EDC) in presence of N-hydroxysuccinimide (NHS). The addition of NHS catalyzed the formation of the intermediate active esters that further react with the amine function of the streptavidin (STV) to yield finally the amide bond between the monoclonal antibody aflatoxin M1 (mAFM1) and the carboxyl group on the nanoparticles.

Characteristic of C=O and N-H stretch analysis was done with various peaks in the FTIR spectrum region shown in Figure 8. However the sharp FTIR absorbance peaks 3426 cm^{-1} specific to amide indicate the presence of proteins.

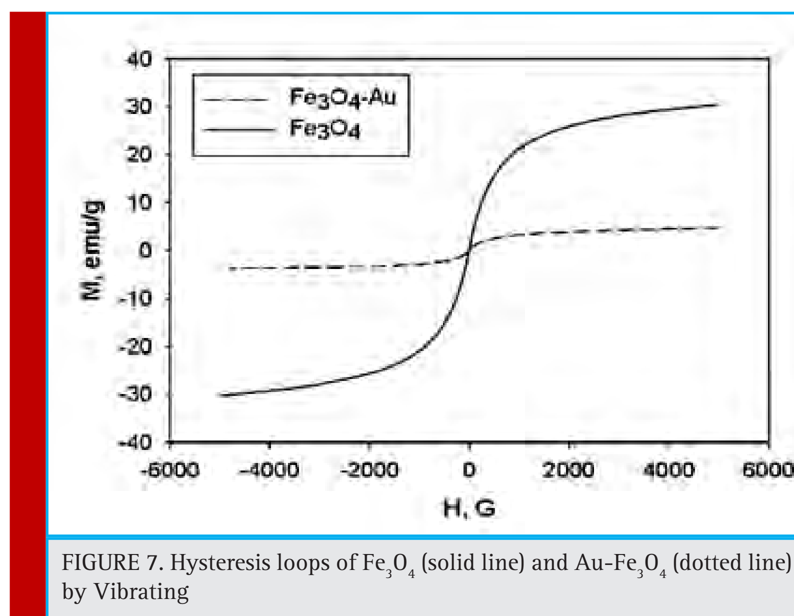


FIGURE 7. Hysteresis loops of Fe_3O_4 (solid line) and Au-Fe_3O_4 (dotted line) by Vibrating

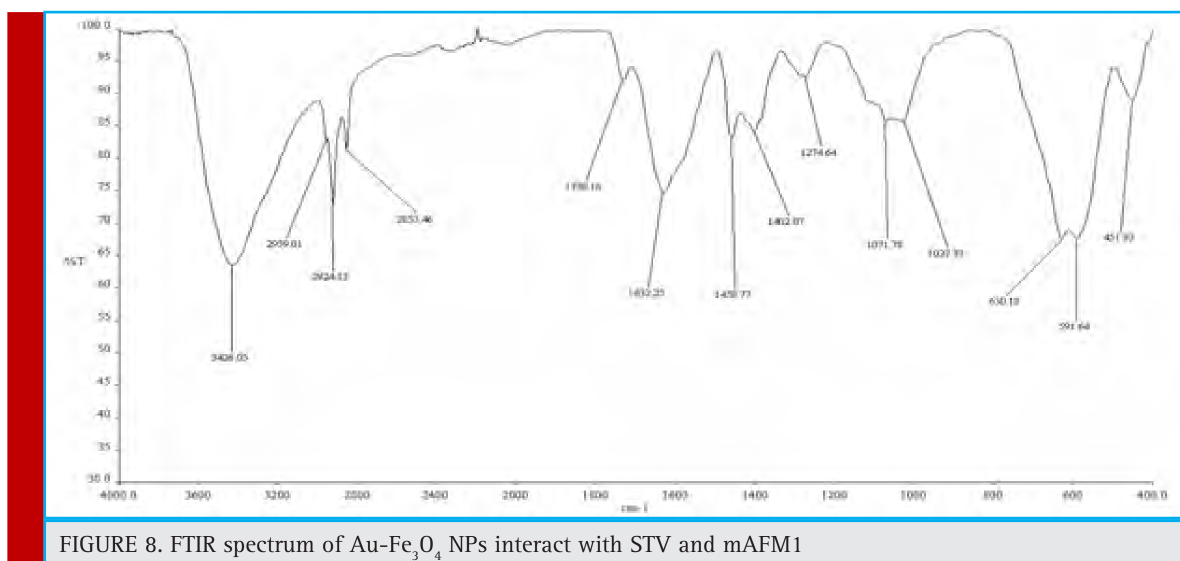


FIGURE 8. FTIR spectrum of Au-Fe_3O_4 NPs interact with STV and mAFM1

The FTIR spectrum in Figure 8 shows peaks at various ranges corresponding to different stretching and bending modes of the amine and carboxylic group. Peaks at 3426 cm^{-1} are assigned to N-H stretching of the amide group. The peak at 1632 cm^{-1} has been assigned to C=O stretching of the carboxylic group. The peak at 3426 cm^{-1} is due to O–H stretching. This type of study has been reported previously to find functional properties, binding and to be used as detection system Adarsha *et al.,* (2015).

DETECTION OF BIOCONJUGATES BY FLUORESCENCE MICROSCOPY

Fluorescence is a process where a fluorophore (FITC) absorb light (492nm) and turns in to an excitation form with apple green color which result in emitted light (515nm). The Au-Fe_3O_4 NPs and its interaction with FITC labeled STV and biotinylated monoclonal antibody of AFM1 (bimAFM1) was measured under fluorescence microscope (Nikon Eclipse N*i*) under fluorescence mode at constant flow of 50 a.u with FITC filter (EX 465-495nm, DM 505 & BA 512-558nm). The background of image became brighter making the particles more difficult to visualize so, it track with the Nikon D-element software.

The fluorescence microscopic image of Figure 9 (A) Au-Fe_3O_4 NPs-FITC labeled STV (B) Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody and (C) Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody-AFM1 bioconjugate was examined under fluorescence and bright field shown in Figure 9. There was no any aggregation found in case of Figure 9 (A) and (B) while in case of (C) shows the aggregation between Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody and AFM1 which indicating the successful bioconjugation. Streptavidin are used ubiquitously due to its remarkable specific binding affinity with biotin which ultimately leads to the aggregation. This aggregation could be exploited to examine at nano level due to ultra-small structure of aflatoxin M1 molecule.

The binding of bimAFM1 antibody and Au-Fe_3O_4 NPs-FITC labeled STV was shown in fluorescence microscopic images. Figure 9 (A), (B) and (C) shows the green fluorescence which indicating the FITC labeled STV binding with Au-Fe_3O_4 NPs, bimAFM1 antibody binding with Au-Fe_3O_4 NPs-FITC labeled STV and aggregation between Au-Fe_3O_4 NPs-FITC labeled STV and bimAFM1 antibody with AFM1. By antigen–antibody reaction anti-AFM1 and aflatoxin M1 developed color by the aggregation of gold nanoparticle conjugate Hoskins *et al.,* (2012). Figure 9 (C) shows the pinkish red color in bright flied microscopy which confirm the aggregation Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody and AFM1.M. Adarsha *et al.,* (2015) has reported the detection of aflatoxin B1 byFe3O4 NPs bioconjugation. Our study is at par to this with slight difference i.e. aflatoxin M1 detection instead of aflatoxin B1.

CONCLUSION

Iron oxide nanoparticles of 10±2 nm has been prepared by the co-precipitation method and in a next step, the nanoparticles have been coated with gold shell of gold it confirmed by super paramagnetic properties of Au-Fe3O4 shows a difference of the magnetization for the coated magnetite nanoparticles in comparison with the uncoated ones. The Fluorescence microscopic characterization shows the successful bioconjugation between Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody and AFM1.

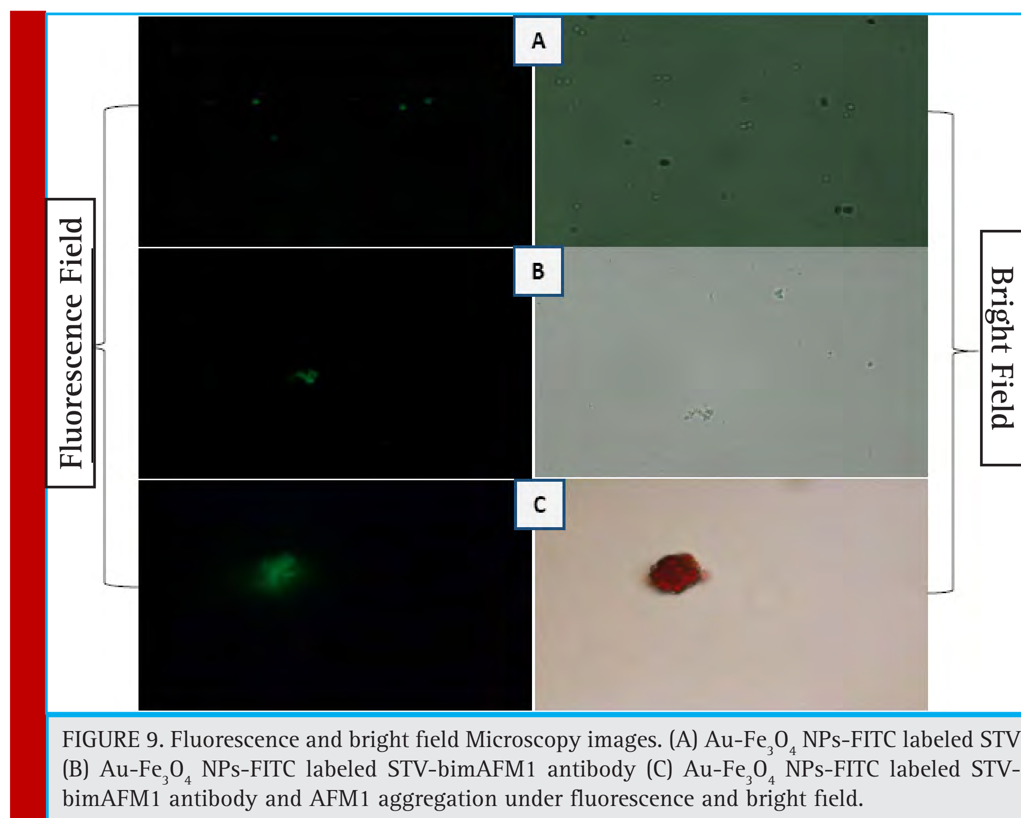


FIGURE 9. Fluorescence and bright field Microscopy images. (A) Au-Fe_3O_4 NPs-FITC labeled STV (B) Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody (C) Au-Fe_3O_4 NPs-FITC labeled STV-bimAFM1 antibody and AFM1 aggregation under fluorescence and bright field.

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Standardization of various factors for production of adventitious roots in selected varieties of *Withania somnifera* and estimation of total withanolides by High Performance Liquid Chromatography

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ABSTRACT

Withania somnifera (Dunal) popularly known as Ashwagandha, "Winter Cherry" and "Indian Ginseng". Its roots and leaves are used in a number of preparations for their anti-inflammatory, anticonvulsive, antitumor properties besides promoting vigour and stamina. Ashwagandha contains very high concentration of metabolites like steroidal lactones (Withanolides), alkaloids and flavonoids, so it is used in more than 200 commercially ayurvedic formulations. The annual requirement of *Withania sominifera* in India is about 9127 MT where as the estimated production in India is only 5905 MT. This requirement can be met by mass cultivation of adventitious roots using bioreactors. Adventitious roots induced by this form are considered to be genetically uniform, true to its type that gives rise to mass production of desired pharmaceutical compound. Seeds of varieties like Jawahar Ashwagandh-20 (JA-20), Arka Ashwagandha (AA), IIHR WS-48 and IIHR WS-32 have been raised in *in-vitro* conditions. Adventitious roots were induced from *in-vitro* leaves by varying factors. Half strength MS medium yielded more roots than full strength MS medium, combination of IAA and IBA (ranging from 0.025-0.01mg/l) were found to be ideal for adventitious root induction for each variety. Sucrose concentration (3-4%) in half strength MS media yielded more adventitious roots, with a light intensity of 16 hours photoperiod than darkness.

KEY WORDS: *WITHANIA SOMNIFERA* (DUNAL), IAA, IBA, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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INTRODUCTION

Withania somnifera (Dunal) popularly known as Ashwagandha, Indian ginseng and Winter cherry belongs to the family *Solanaceae*. The plant's Latin name literally means, "sweat of a horse" due to the scent of the roots. The plant is commonly found in Africa, Mediterranian, India and North America. It is an erect branched under shrub up to 1.25 m in height, minute, smooth and shiny hairs throughout the plant. Leaves are ovate, with hairs and soft to touch. Flowers are greenish, the roots are fleshy, papery and whitish brown in color. The stems are around 3 to 4 feet in height. One plant survives for up to 4 to 5 years. Its stem contains fiber like texture. The leaves are oval shaped, 2 to 4 inches long and contain fiber. The flowers are blooming at the base of the stems are small, somewhat long with chimney shape and yellowish green in color. The flowers bloom from the base of the leaves and become red when wipe. The seeds are small, heart shaped, smooth and flat. The roots are rough, white from within, strong, transparent, thick and one to one and half feet long, (Geetha et al., 2018).

In Ayurveda and Unani medications the herb is majorly used for its high rejuvenating power. It is called as "The natural stress buster" due to its ability in making the human body copes with different kinds of stress (Rao, 2012). The roots of Ashwagandha help in boosting immunity power of the body. It is commonly prescribed for hiccup, bronchitis, dropsy, rheumatism and female disorders, the roots of this plant also prescribed for general sexual weakness in human beings (Kattimani *et al.*, 2000). Its roots and leaves are used in a number of preparations for their anti-inflammatory, anticonvulsive, antitumor, immuno-suppressive and antioxidant properties besides promoting vigor and stamina. Ashwagandha is increasingly becoming a popular adaptogenic herb and is available throughout the western world as a dietary supplement. Ashwagandha contains very high concentration of metabolites like steroidal lactones (Withanolides), alkaloids and flavonoids, so it is used in more than 200 commercially ayurvedic formulations.

Adventitious roots are the roots that are induced at unusual sites such as roots forming on leaves, which grow and branch rapidly (Dubrovsky and Rost., 2003). The roots induced by this form are considered to be genetically uniform, true to its type, that gives rise to mass production of desired pharmaceutical compound (Goel *et al* ., 2009). Adventitious root cultures provide a preferred platform to produce commercially important secondary metabolites (Khan *et al* 2017). Adventitious roots can harbor medicinally important compounds through different strategies like elicitation, temperature stress etc., (Rani *et al.*, 2017).

The technique of micro propagation is applied with the objective of enhancing the rate of multiplication. Through the culture over a million of plants can be grown from a small piece of plant tissue within 12 months. Such proliferative rate of multiplication cannot be expected by any *in-vivo* methods. Large scale production through plant *in-vitro* regeneration will provide a means of putting the plant onto the market at lower prices. In addition, the technique is cost effective, relatively simple and can be performed by semi-skilled persons. A sustained supply of the source material often becomes difficult due to the factors like environmental changes, cultural practices, diverse geographical distribution, labour cost, selection of the superior plant stock, over exploitation by pharmaceutical industry (Kaur et al 2017)

Optimization of various tissue culture techniques become very important to explore *W. somnifera* at different aspects, as plants obtained from fields fileds are not enough for all in vitro studies. Therefore, efficient tissue culture techniques like, micropropaogation, regeneration, organogenesis, hairy root production, etc. have been established, (Vibha pandey *et al.* 2017).

The requirement of dried plant material for withanolides drug production in India is estimated to be 9127 tonnes against the annual production of 5905 tonnes (Sharda *et al.*, 2007). Moreover, field cultivation is time consuming, laborious and not able to meet the Ashwagandha global market requirement (Sivanandan *et al.*, 2012b; 2013a). To improve the commercial cultivation of Ashwagandha, biological advances must be made that should either increase yield or reduce time gap to assure quality (Banerjee *et al.*, 1994).

The provision of alternative sources of *Withania somnifera* by encouraging its cultivation will go a long way in reducing their heavy dependence on the wild populations and also major diseases of plant like seed rot and blight can be overcome. The main objective of this research is to develop a reproducible protocol for adventitious root induction from in-vitro leaves, and comparative analysis of withanolides present in in vitro adventitious roots roots of different varieties and selection of best variety for mass propagation in bioreactors.

MATERIALS AND METHODS

Selection and establishment of plant material for micropropogation.

Seeds of four high yielding varieties of *Withania somnifera* like JA-20 (released variety from MPKVV, Madhya Pradesh) used as check in AICRP national trials, Arka Ashwagandha (released variety from IIHR, Bangalore), IIHR WS-32 and IIHR WS-48 were selected for the experiment.

The seeds were soaked in 300 ppm of gibberellic acid for 12 hours and washed with water. The seeds were

pre-treated using 100 mg Dithane M-45 fungicide for 15 minutes, washed with sterile water followed by 70%(v/v) ethanol for 1 minute, washed with sterile water and then with 0.1% (w/v) sodium hypochlorite(5% (w/v) available chlorine, NICE Kochi Solution) for 4 minutes. Then seeds were washed with sterile double distilled water 2-3 times to remove traces of sodium hypochlorite and dried. The seeds were then inoculated into half strength MS medium (Murashige and Skoog , 1962).

Procedure for adventitious root induction

Leaves from two month old explants were taken for production of adventitious roots, before inoculation, leaves were cut in middle with sterilized scalpel and placed on MS culture medium with the adaxial surface down. Different factors were varied–

1. Strength of medium: Half strength and full strength MS medium with selected combination of auxins were used to study adventitious root induction.
2. Sucrose concentration: Carbohydrate source plays an important role in maintaining osmoticum in plant tissue culture. Root initiation and development is a high energy process which requires the expense of available metabolic substrates such as sugars. Sucrose at different concentrations (2-7%) were tried.
3. Auxin treatment: In order to determine the optimal conditions for adventitious root induction, we tested various concentration of auxins (IAA and IBA) ranging from 0.025-0.01mg/L in 5 different combinations and compared it with control (without IAA and IBA).
4. Light intensity: The cultures in half strength MS medium with selected auxin combination were incubated at 25±2°C with 16 hours photoperiod under cool fluorescent light and for dark treatment the bottles were placed in shelves without light.

   The adventitious roots were observed after 15 days and parameters like number of roots per explant and percentage of explants response for root induction were studied.

   The roots were subjected for HPLC analysis to estimate the total withanolide content.

5. Extraction of bioactive principles from *W.somnifera*

The adventitious roots extracted from *in-vitro* were washed twice with milli-Q water to remove the traces of agar, dried and powder dried using pestle and mortar. They were assessed for different components that contribute to total withanolides. The analysis was carried out by HPLC method (Agarwal and Murali, 2010). Two grams of dry root powder was extracted with 50 mL of methanol on boiling water bath for about 20 minutes and transfer the extract to a 250 mL beaker. Repeat the process 3-4 times till the extract was colorless. Then collected all the extracts and made up the volume to 100mL with methanol, mixed well and filtered through 0.45 micron membrane filter and these were subjected to analysis by HPLC with Photo Diode Array detector. Seven standards such as Withanoside IV, Withanoside V, Withaferine A, Withanolide A, Withanolide B, 12- deoxy Withanostramolide and Withanone were used to quantify the amount of various withanolides present in the root samples. Chromatogram was recorded at 227nm wavelength and later calculate the contents of individual withanolides by the using the formula and expressed as mg/100g dry weight basis.

Area of the sample ×
Standard Wt. (mg) ×
Sample dilution ×
Purity of standard
------------------------- × 100
Area of the standard ×
Standard dilution ×
sample weight (mg) × 100

RESULTS AND DISCUSSION

The results from the present study demonstrated that standardization of different factors like strength of the medium, effect of photoperiod, sucrose concentration , combinations of auxins at different concentrations is essential for effective adventitious root induction in *Withania somnifera*.

In this study to determine the effects of media strength half strength MS medium with a combination of 0.25mg/l IAA and 0.75 mg/l IBA had higher number of induction response and also higher number of roots per explants (Table no 1 and 2). The results of the present study is similar to results of the previous studies on adventitious root induction in *Withania somnifera* by Wadegaonkar *et al* (2006) and Praveen and Murthy (2010), where half strength MS medium was chosen suitable for adventitious root induction. It contradicts with Yin *et al* (2013) induced adventitious roots in *Pseudostellaria heterophylla* in full strength MS medium with 3mg/L IBA using root explants.

Highest root induction response (98.2% in JA 20) and highest number of roots per explant (16 roots in WS 32) was observed in bottles placed under 16 hours photoperiod compared to darkness in all the varieties of *Withania somnifera* (Table 3 and 4). Explants incubated under darkness induced profuse callusing which subsequently turned brownish and hindered the induction of roots. Roots initiated were thick and longer in braches under 16 hours photoperiod compared to thin and brittle roots in darkness.

Table 1. Effect of strength of the medium on the induction response (%)				
Treatment	**Arka Ashwagandha**	**JA 20**	**IIHR WS 32**	**IIHR WS 48**
Half MS	98.00±0.173a	98.00±0.520a	98.60±0.387a	97.40±0.316a
Full MS	95.4±0.173b	96.20±0.520b	96.40±0.387b	96.40±0.316b

Table 2. Effect of strength of the medium on number of roots per explant				
Treatment	**Arka Ashwagandha**	**JA 20**	**IIHR WS 32**	**IIHR WS 48**
Half MS	13.80±0.070a	13.60±0.173a	14.20±0.173a	12.60±0.141a
Full MS	13.20±0.070b	13.20±0.173b	13.80±0.173b	12.40±0.141b

Table 3. Effect of photoperiod on adventitious root induction								
Treatments	**Induction Response (%)**				**No of roots per explant**			
	AA	JA 20	WS 32	WS 48	AA	JA 20	WS 32	WS 48
16 hr photoperiod	97.8	98.2	97.6	97.6	13.5	15.25	16	16.45
Darkness	91.8	91.6	92	91.8	5.1	5.1	5.05	5.6
S.Em	0.2151				0.245			
CD 5%	0.619				1.75			
CD 1%	0.81				2.36			
CV	0.717%				4.69%			

Table 4. Effect of photoperiod on number of days taken for adventitious root induction				
Treatment	**Arka Ashwagandha**	**JA 20**	**IIHR WS 32**	**IIHR WS 48**
16 hours Photoperiod	24.00±0.316a	26.00±0.346a	26.60±0.141a	26.00±0.346a
Darkness	15.00±0.316b	14.80±0.346b	15.80±0.141b	14.80±0.346b

Praveen and Murthy (2010) also established adventitious roots from leaf segments of *Withania somnifera* on half strength MS medium(0.8%) agar with 0.5mg/L IBA, 30g/L sucrose incubated under 16 hours photoperiod with 100% of explants response for root induction.

Table 1- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at P<0.05 according to Duncan multiple range test in all tables Data were scored after15 days of culture

Table 2- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at P<0.05 according to Duncan Range Multiple Test. Data were scored after 15 days of culture

Table 5. Effect of Sucrose concentration on adventitious root induction response (%)				
Treatments (Induction response %)	**Arka Ashwagandha**	**JA 20**	**WS 32**	**WS 48**
2% Sucrose	88.00±0.397b	82.40±0.465 b	80.40±0.389 b	88.20±0.499b
3% Sucrose	96.60±0.397a	86.80±0.465a	85.80 ±0.389a	98.40±0.499a
4% Sucrose	79.40±0.397c	78.60±0.465c	75.40±0.389c	77.00±0.499c
5% Sucrose	61.40±0.397d	61.60±0.465d	61.20±0.389 d	61.00 ±0.499 d
6% Sucrose	33.60±0.397e	35.60±0.465e	31.60±0.389 e	34.60±0.499 e
7% Sucrose	10.20±0.397f	11.00±0.465f	10.20 ±0.389f	10.60 ±0.499f

Table 6. Effect of Sucrose concentration on number of adventitious roots per explant

Treatments	Arka Ashwagandha	JA 20	WS 32	WS 48
2% Sucrose	11.40±0.319c	11.80±0.331c	10.60±0.294c	11.80±0.261c
3% Sucrose	17.60±0.319a	17.20±0.331a	15.00±0.294a	17.20±0.261a
4% Sucrose	12.80±0.319b	14.00±0.331b	12.40±0.294b	14.20±0.261b
5% Sucrose	8.80±0.319d	10.20±0.331d	9.8 ±0.294c	10.40±0.261d
6% Sucrose	7.40±0.319e	7.60±0.331e	8.60±0.294e	7.40±0.261e
7% Sucrose	5.40±0.319 f	5.60±0.331f	5.20±0.294e	5.40±0.261f

Table 7. Effect of auxins on adventitious root induction response (%)

Treatments	Arka Ashwagandha	JA 20	WS 32	WS 48
0 IAA+0 IBA	0.000±0.284f	0.000±0.330f	0.000±0.614f	0.000±0.703
0.25 IAA+0.75 IBA	96.60±0.284a	97.20±0.330a	85.80±0.614b	98.40±0.703
0.5 IAA+0.5 IBA	80.80±0.284c	80.60±0.330c	80.40±0.614c	82.20±0.703
0.75 IAA+0.25 IBA	76.80±0.284d	77.40±0.330d	75.60±0.614d	75.00±0.703
0 IAA+1 IBA	83.6±0.284 b	82.40±0.330b	97.6±0.614a	85.60±0.703
1 IAA+0 IBA	72.4±0.284e	68.00±0.330e	67.80±0.614e	65.00±0.703e

Table 8. Effect of auxins on number of adventitious roots per explant

Treatments	Arka Ashwagandha	JA 20	WS 32	WS 48
0 IAA+0 IBA	0.000±0.257d	0.000±0.245e	0.000±0.238f	0.000±0.371e
0.25 IAA+0.75 IBA	17.60±0.257a	17.20±0.245a	15.00±0.238b	17.20±0.371a
0.5 IAA+0.5 IBA	13.80±0.257b	13.80±0.245b	14.20±0.238c	12.20±0.371c
0.75 IAA+0.25 IBA	11.60±0.257c	11.60±0.245c	11.60±0.238d	13.00±0.371bc
0 IAA+1 IBA	13.60±0.257b	14.40±0.245b	17.40±0.238a	13.60±0.371b
1 IAA+0 IBA	11.40±0.257c	9.20±0.245d	8.80±0.238e	9.80±0.371d

Table 3- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Data were scored after 15 days of culture. Growth Conditions- Media- Half MS supplement with selected auxin combination, photoperiod-16 hours, culture period-3 weeks at 25±2°C

Table 4- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at $P<0.05$ according to Duncan Range Multiple Test. Data were scored after 15 days of culture. Growth Conditions- Media- Half MS supplement with selected auxin combination, photoperiod-16 hours and complete darkness, culture period-3 weeks at 25±2°C

Carbohydrate plays an important role in maintaining osmoticum in plant tissue culture. Sucrose is considered as an unquestionably important carbon and energy source which is found in abundance in phloem sac involved in developmental process. From the observations of the above study, the optimal condition for adventitious root induction in *Withania somnifera* was half strength MS medium with 3% sucrose concentration, (Table 5 and 6).

It has been documented earlier that a sucrose concentration (3 %) was suitable for hairy root growth, whereas a too low or too higher a concentration of sucrose was adverse to adventitious root growth in *W. somnifera* (Sivanandhan *et al.* 2012 a). A lower concentration cannot provide enough energy and therefore may not be able to act as building blocks. However, higher sucrose concentration exhibited negative effect in growing cells. Nagella and Murthy (2010) recorded that 3 % sucrose was suitable for biomass accumulation and withanolide A production in cell suspension culture of *W.somnifera*. Sucrose at higher concentrations in the nutrient medium normally reduces cell biomass due to the increase of osmotic potential which subsequently reduces the uptake of nutrients. A similar result was obtained by Zhang *et al.* (2012) in *Periploca sepium* adventitious root culture and by Sivanandhan *et al.* (2012 a) in *W. somnifera* adventitious root and hairy root cultures.

Table 9. Analysis of bioactive principles from *W. sominifera* using HPLC.

Variety names	Withanoside IV	Withaferin A	Withanolide A	Withanolide B	Total Withanolide
Arka Ashwagandha	0.061±0.001 a	0.023±0.000a	0.000±0.000b	0.000±0.000b	0.084±0.001a
JA 20	0.018±0.001d	0.018±0.000c	0.002±0.000a	0.005±0.000a	0.043±0.001d
IIHR WS 32	0.0034±0.001b	0.022±0.000b	0.000±0.000b	0.000±0.000b	0.057±0.001b
IIHR WS 48	0.030±0.001c	0.017±0.000b	0.000±0.000b	0.000±0.000b	0.047±0.000c

The lowest induction response was observed in 7% sucrose concentration, from this result it was noticed that the number of roots per explant started decreasing at a higher root concentration of 5% and above. Higher amount of sucrose can retard the development of cultured cells (Wu et al., 2006) by causing cessation of the cell cycle when other nutrients are limited (Gould et al., 1981).

Table 5- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at P<0.05 according to Duncan Range Multiple Test. Data were scored after15 days of culture. Growth Conditions- Media- Half MS supplement with selected auxin combination, photoperiod-16 hours, culture period-3 weeks at 25±2°C

Table 6- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at P<0.05 according to Duncan Range Multiple Test. Data were scored after15 days of culture. Growth Conditions- Media- Half MS supplement with selected auxin combination, photoperiod-16 hours, culture period-3 weeks at 25±2°C.

Development of roots or shoots from explants involved in organogenesis depends on morphogenetic potentiality of the cells. Dedifferentiation, induction of organogenesis pathway and development of organ are the three distinct stages during organogenesis (de Kler *et al*., 1997). Supplementation of exogenous auxin is essential for adventitious root development (Pop *et al*., 2011). IAA and IBA induced adventitious roots from leaf explants after 12 days of culture. Protuberances developed in leaf explants within a week from the cut ends and adventitious roots directly developed from these protuberances in another week. The percentage of explants response for root induction and number of roots initiated per explants were recorded after 3 weeks of culture.

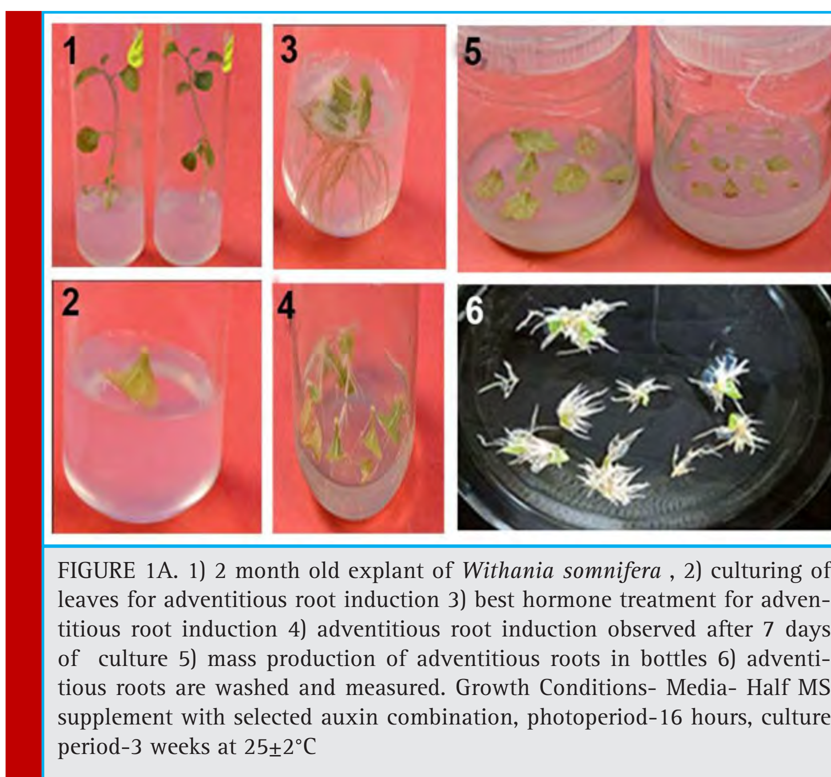

FIGURE 1A. 1) 2 month old explant of *Withania somnifera* , 2) culturing of leaves for adventitious root induction 3) best hormone treatment for adventitious root induction 4) adventitious root induction observed after 7 days of culture 5) mass production of adventitious roots in bottles 6) adventitious roots are washed and measured. Growth Conditions- Media- Half MS supplement with selected auxin combination, photoperiod-16 hours, culture period-3 weeks at 25±2°C

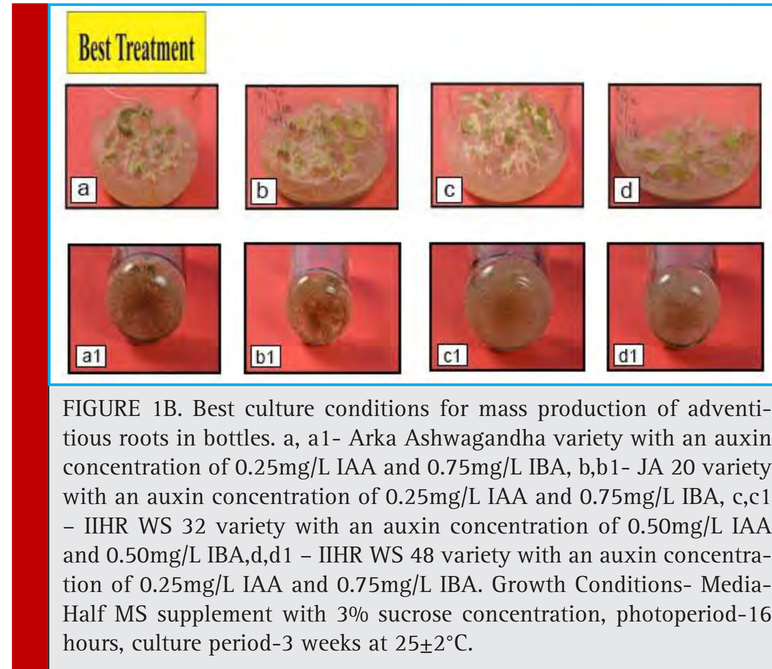

FIGURE 1B. Best culture conditions for mass production of adventitious roots in bottles. a, a1- Arka Ashwagandha variety with an auxin concentration of 0.25mg/L IAA and 0.75mg/L IBA, b,b1- JA 20 variety with an auxin concentration of 0.25mg/L IAA and 0.75mg/L IBA, c,c1 – IIHR WS 32 variety with an auxin concentration of 0.50mg/L IAA and 0.50mg/L IBA,d,d1 – IIHR WS 48 variety with an auxin concentration of 0.25mg/L IAA and 0.75mg/L IBA. Growth Conditions- Media- Half MS supplement with 3% sucrose concentration, photoperiod-16 hours, culture period-3 weeks at 25±2°C.

Taiz and Zeiger (2002) reported that roots may require a less concentration of auxin to grow, but root growth is strongly inhibited by its higher level because at this level, auxin induces the production of ethylene, a root growth inhibitor. The adventitious roots were also induced in leaf explants of *Withania somnifera* using a combination of IAA and IBA by Praveen and Murthy (2010). Combination of IBA and IAA performed better than individual treatment of auxin upon adventitious root induction in *Withania somnifera* (Sivanandan *et al* 2012a). Wadegoankar *et al* (2006) reported that a combination of IAA and IBA was effective in adventitious rot induction in leaves of *Withania somnifera* .

Hence the best auxin concentration for adventitious root induction with 96.6% root induction with 18.25 roots per explant was observed in Arka Ashwagandha variety with an auxin concentration of 0.25mg/L IAA and 0.75mg/L where as a combination 0.25mg/L IAA and 0.75mg/L IBA yielded in 97.2% root induction response and 15.12 roots per explant in JA 20. Induction response of 97.2% with 18.5 roots per explant was observed in an auxin concentration 0.25mg/L IAA and 0.75mg/L IBA of in IIHR WS 48 and induction response of 97.6% with 18.38 roots per explant was observed in explants inoculated into half strength MS medium with 0.50 mg/L IAA and 0.50 mg/L IBA for IIHR WS 32.

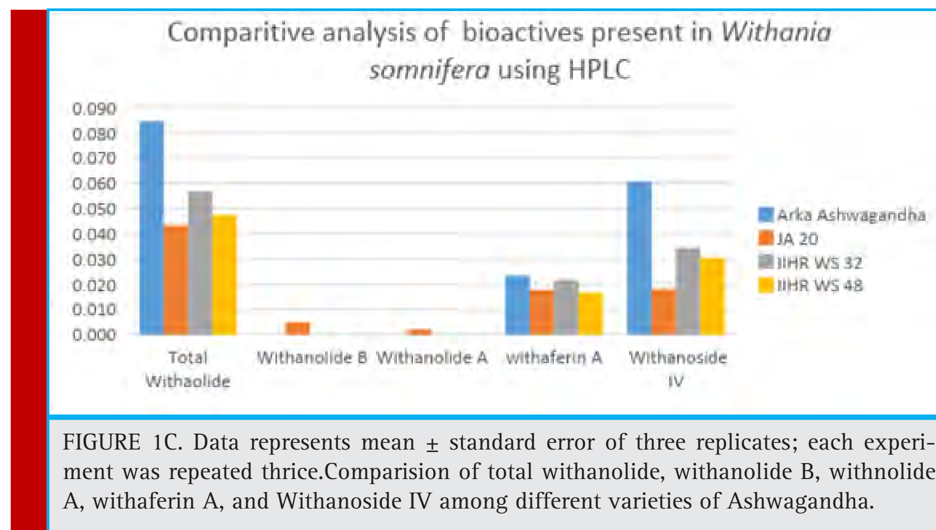

FIGURE 1C. Data represents mean ± standard error of three replicates; each experiment was repeated thrice.Comparision of total withanolide, withanolide B, withnolide A, withaferin A, and Withanoside IV among different varieties of Ashwagandha.

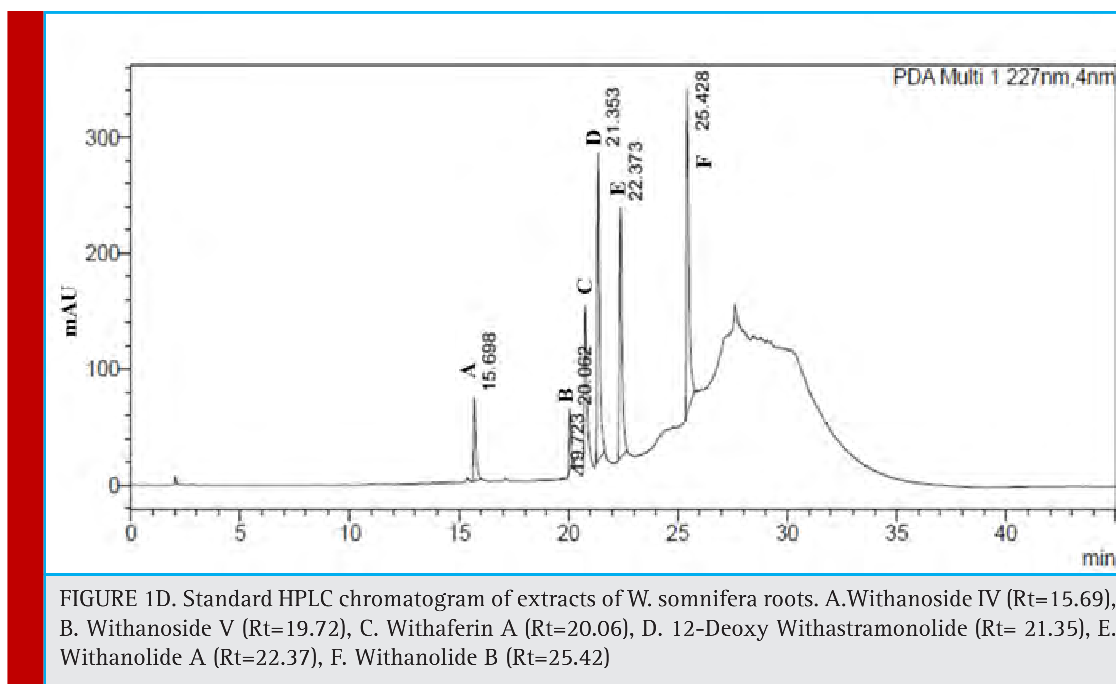

FIGURE 1D. Standard HPLC chromatogram of extracts of W. somnifera roots. A.Withanoside IV (Rt=15.69), B. Withanoside V (Rt=19.72), C. Withaferin A (Rt=20.06), D. 12-Deoxy Withastramonolide (Rt= 21.35), E. Withanolide A (Rt=22.37), F. Withanolide B (Rt=25.42)

(Table 7 and 8), At the end of 15 day after explant inoculation, the length of the root was between 0.5-1.0 cm and it was observed that the root length steadily increased with the increase in growth period with was approximately 4-5 cm after 30 days. These results indicate fast growing nature of adventitious roots.

Table 7-Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at P<0.05 according to Duncan Range Multiple Test. Data were scored after15 days of culture. Growth Conditions- Media- Half MS supplement with selected auxin combination, photoperiod-16 hours, culture period-3 weeks at 25±2°C.

Table 8- Values are mean ± standard error of five replications in three independent experiments, each with three explants per treatment. Means followed by the same letter are not significantly different at P<0.05 according to Duncan Range Multiple Test. Data were scored after15 days of culture. Growth Conditions-Media- Half MS supplement with selected auxin combination, photoperiod-16 hours, culture period-3 weeks at 25±2°C.

Variation persist in accumulation of withanolides due to plant parts, developmental stages (Praveen and Murthy, 2010), plant part obtained from different types of cultures (Sharada *et al.*, 2007; Singh *et al.*, 2017) of *W. somnifera*. These studies establish relationship between morphology/condition of plant tissue and withanolide contents. Sivanandhan et al., 2012b, 2013b; Singh *et al.*, 2017) used in vitro grown plants in different studies to develop adventitious roots, using different growth conditions. These developed roots were harvested to extract different combinations of withanolides.

The results for total withanolides analyzed in adventitious roots of four genotypes revealed significant difference among them (Table 9).

Among the genotypes, Arka Ashwagandha recorded high total withanolide content of 0.084% when compared to check JA-20 (0.043%). Among seven withanolides analyzed, withanoside V, 12- deoxy Withanostramolide and Withanone were not detected in all genotypes and withanoside IV constitutes highest in all the genotypes. Withanolide A and B were detected only in JA-20. Arka Ashwagandha and IIHR WS-32 contain high withaferin A and Withanoside IV when compared to Check JA-20.

Table 9-Values are mean ± standard error of three repeated experiments, each experiment was repeated thrice. Means followed by the same letter are not significantly different at P<0.05 according to Duncan Range Multiple Test.

CONCLUSION

From the present research a standard protocol has been developed for mass production of adventitious roots from *in-vitro* leaves in *Withania somnifera*. A variety with highest total withanolide content has been identi-

fied in comparision to JA 20. The requirement of dried plant material for withanolides drug production in India is high. Moreover, field cultivation is time consuming, laborious and not able to meet the Ashwagandha global market requirement. By transferring these roots in to suspension culture and mass propagating it in bioreactors reduce time gap compared to field grown roots and assure good quality *Withania somnifera* roots with high total withanolide content to cater the global demand. Large scale production through plant *in vitro* regeneration will provide a means of placing the plant onto the market at lower prices. In addition, the technique is cost effective, relatively simple and can be performed by semi-skilled persons.

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Insights into the antagonism of *Lactobacillus fermentum* curd isolate against Gram-positive and Gram-negative pathogenic bacteria

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ABSTRACT

This communication characterizes the curd isolate of lactic acid bacteria having the capacity to antagonize human pathogenic bacteria. The commercially available curd, in sealed form in a plastic cup, was procured from Malda town market (West Bengal state, India) and processed microbiologically, using de Man Rogosa Sharpe medium, for the isolation of lactic acid bacteria. The pure bacteria culture obtained was identified, by phenotypic characterization through conventional methods, as *Lactobacillus fermentum*, and designated as LMEM 22. The *Lactobacillus fermentum* LMEM 22 curd isolate had mixed antibiotic susceptibility patterns, showing resistance (ZDI: ≤15 mm) to amikacin, ciprofloxacin, kanamycin, methicillin and vancomycin, sensitivity (ZDI: ≥ 21 mm) to ampicillin, amoxyclav, gentamycin, cefotaxime, imipenem, meropenem and tetracycline, and intermediate susceptibility (ZDI: 16 – 20 mm) to cfoxitin and trimethoprim. The *L. fermentum* LMEM22 antagonizes both gram-negative: *Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Salmonella enterica serovar* Typhi, and gram-positive: *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes*, bacterial pathogens, following agar overlay (ZDI: 17±1.73 mm to 20±1.00 mm, for gram-positive, and 18±2.00 mm to 33±2.65 mm for gram-negative bacteria) as well as agar-well diffusion (ZDI: 10.67±2.08 mm to 12±1.00 mm, for gram-positive, and 13.00±2.65 mm to 18.00±3.00 mm, for gram-negative bacteria) techniques. The overall bacteriocin activity (AU/ml) of *Lactobacillus fermentum* LMEM 22 for the test bacterial pathogens ranged 142.27 – 240.00, and the 'R' value ranged 5.5 – 13.5. This study underlines the usefulness of locally available lactic acid bacteria in designing the probiotic microorganisms for biotherapy.

KEY WORDS: LACTIC ACID BACTERIA, *LACTOBACILLUS FERMENTUM*, ANTAGONISTIC ACTIVITY, BACTERIOCIN ACTIVITY, PATHOGENIC BACTERIA

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INTRODUCTION

Among the lactic acid bacteria (LAB), *Lactobacillus* spp. are characteristically known as probiotics, meaning, as per the definition of FAO/WHO (2001), the 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'; LAB isolates are beneficial in many ways, but, essentially by restricting the toxigenic bacterial growth in the gut (Podolsky, 1998). The FAO/WHO (2007) suggested that the probiotic microorganisms must possess the capacity to display the antagonistic activity against bacterial pathogens. Among the large number of lactobacilli isolated from various fermented foods, 42 isolates showed activity against *Escherichia coli*, while 15 isolates had antibacterial activity against *Klebsiella pneumoniae* (Shehata *et al*., 2016).

Nivien *et al.* (2016) isolated LAB from fermented milk, identified the isolates by phenotypic characterization and reported their antibacterial activity against the bacterial strains: *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. It has been reported that the lactobacilli, including *Lactobacillus fermentum*, had growth inhibitory action against gram-positive as well as gram-negative human pathogenic bacteria (Vuotto *et al*., 2016). As per the report of Sharma *et al.* (2016), the LAB (*Pediococcus acidilactici* and *Lactobacillus casei*) isolated from milk cream and lassi had growth inhibitory activity against a number of gram-positive food borne bacteria.

Benavides *et al.* (2016) demonstrated that the *Lactobacillus fermentum* isolate from local ecological niche was sensitive to ampicillin, cefuroxime, tetracycline and amoxicillin/clavulanic acid and resistant to gentamycin and kanamycin, and the LAB was found inhibitory to *Escherichia coli* and *Salmonella* Typhimurium. As per the previous report (Halder *et al*., 2017), four lactobacilli (*Lactobacillus animalis* LMEM6, *Lactobacillus plantarum* LMEM7, *Lactobacillus acidophilus* LMEM8 and *Lactobacillus rhamnosus* LMEM9) procured from different commercially available curd samples had antibacterial activity against gram-negative pathogenic bacteria, such as *Escherichia coli*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhi, and had resistance to vancomycin and amoxyclav.

Recently, Mahalot and Mandal (2018) have isolated LAB from locally available cow milk and goat milk samples showing sensitivity to most of the test antibiotics, while resistance was recorded for all isolates to methicillin, for *Lactobacillus* sp. G1 and *Lactococcus* sp. G2 to trimethoprim, while to vancomycin for *Lactobacillus* sp. G1 and *Lactobacillus* sp. C1. Since the good LAB are not even waived from antibiotic resistance phenomenon, many authors documented the status of various resistances to antibiotics for safety profiling of native LAB isolates (Mandal *et al*., 2017). This background prompted us to assess the broad spectrum antibacterial activity of lactic acid bacillus isolated from commercially available curd, and explore the antibiogram of the isolated *Lactobacillus*, through phenotypic characterization.

MATERIAL AND METHODS

A single cup of commercially available curd sample was procured from Malda town market (West Bengal, India), and processed microbiologically for the isolation of lactic acid bacteria (LAB), following the protocol mentioned earlier (Halder and Mandal, 2015): growth enrichment of LAB in MRS broth (Hi-Media, India), pure culture (single discrete colony isolation) of LAB on MRS agar (Hi-Media, India) plate, and storage of the LAB in MRS agar stab at 4°C for further processing. The isolated LAB (n=1), following Bergey's manual (Holt, 1984), as described earlier (Halder and Mandal, 2015), was subjected to phenotypic (gram-staining, colony morphology study and motility test) and biochemical (oxidase and catalase production) characterization. The non-motile non-spore forming gram-positive rod shaped bacteria (no cocci were found), showing negative results to oxidase and catalase tests, were subjected to IMViC, amino acid decarboxylation and sugar fermentation tests.

The antibiotic susceptibility of the LAB (LMEM 22) was executed by disc diffusion method (Bauer *et al*., 1996), the details of which was described before (Halder and Mandal, 2016; Halder *et al*., 2017). The antibiotic discs (Hi-Media, Mumbai, India) used in the study included amikacin (Ak: 30-µg/disc), amoxyclav (Ac: 30-µg/disc), ampicillin (Am: 10-µg/disc), ciprofloxacin (Cp: 5-µg/disc), cfoxitin (Cx: 30-µg/disc), cefotaxime (Ct: 30-µg/disc), cefotaxime/clavunilic acid (Cc: 30/10-µg/disc), gentamycin (Gm: 30-µg/disc), imipenem (Ip: 10-µg/disc), kanamycin (Km: 30-µg/disc), methicillin (Me: 5-µg/disc), meropenem (Mp: 10-µg/disc), tetracycline (Tc: 30-µg/disc), trimethoprim (Tm: 5-µg/disc) and vancomycin (Vm: 30-µg/disc). The results, in terms of ZDI (zone diameter of inhibition) values, were interpreted according to Liasi *et al.* (2009) and Vlkova *et al.* (2006), in order to label the test bacterial isolate as resistant (ZDI: ≤ 15 mm), sensitive (ZDI: ≥21 mm), or intermediately susceptible (ZDI: 16–20 mm).

The antagonistic activity of the LAB LMEM 22 isolate from curd was determined against gram-negative (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhi) and gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis, Listeria monocytogenes*) bacterial pathogens,

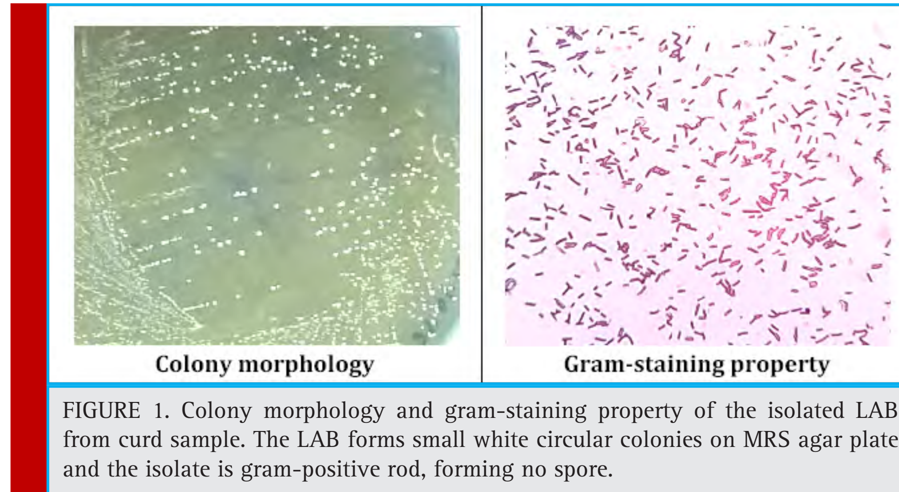

FIGURE 1. Colony morphology and gram-staining property of the isolated LAB from curd sample. The LAB forms small white circular colonies on MRS agar plate and the isolate is gram-positive rod, forming no spore.

following agar-well diffusion (Tagg, 1971; Halder *et al.*, 2017) and agar overlay (Shokryazdan *et al.*, 2018) methods, as described and interpreted earlier (Shokryazdan *et al.*, 2014, Halder *et al.*, 2017, Mandal and Halder, 2018).

The 'R' values, from the action of LMEM 22 isolate over the bacterial pathogens, were calculated applying the formula described elsewhere (Halder and Mandal, 2016), and interpreted according to the criteria mentioned earlier (Carasi *et al.*, 2014; Pisano *et al.*, 2014), while the bacteriocin activity of LAB (LMEM 22), in terms of arbitrary units per milliliter (AU/ml), was calculated following the formula put forwarded by Iyapparaj *et al.* (2013).

RESULTS AND DISCUSSION

As has been demonstrated by Iyapparaj *et al.* (2013), the morphologically identical bacterial colonies, procured from goat milk on the MRS agar plate, have been identified as *Lactobacillus* sp., on the basis of physical and biochemical characteristics, following Holt *et al.* (1984). In this study, a single isolate of non-motile non-spore forming gram-positive rod was procured from the curd sample (Figure 1), and the isolated bacteria (LMEM 22) was an hetero-fermentative strain, which in TSI test showed the production of acid as well as gas (CO_2). The LMEM 22 isolate showed negative test results for catalase and oxidase, and in IMViC test battery the isolates was positive for methyl red. The sugar fermentation pattern of LMEM 22 isolate is represented in Table 1, while the amino acid decarboxylation test results are depicted in Figure 2. Thus, following phenotypic and biochemical characterization the isolated LAB was identified as *Lactobacillus fermentum* LMEM 22. The LAB isolates procured from different fermented foods, including curd, have been identified earlier by Nigam *et al.* (2012), following phenotypic characterization of the bacteria. Currently, the treatment options with antibiotics are inadequate because of the escalating rate of emergence of antibiotic resistant pathogenic bacteria causing life-threatening infections to humans. Alternative to the antibiotics, which remains the mainstay of all therapy for bacterial infections (Van Boeckel *et al.*, 2014), probiotic lactobacilli have been found suitable for biotherapy with proven antibacterial activity (Iyapparaj *et al.*, 2013; Sing *et al.*, 2017).

Earlier, it has been reported that the curd isolates of *Lactobacillus animalis* LMEM6, *Lactobacillus plantarum* LMEM7, *Lactobacillus acidophilus* LMEM8 and *Lactobacillus rhamnosus* LMEM9, had bacterial growth inhibitory activity, having ZDIs 13.67 ± 0.58 – 29.50 ± 2.10 mm, by agar-well, and 11.33 ± 0.58 – 35.67 ± 2.52, by agar overlay, against human pathogenic bacteria, viz., *Escherichia coli*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhi (Halder *et al.*, 2017). The antibacterial activity of *Lactobacillus fermentum* LMEM 22 against gram-positive and gram-negative pathogenic bacteria, following agar-well diffusion method is depicted in Figure 3.

Gandevia *et al.* (2017) isolated, from cow milk, buffalo milk, goat milk and curd samples, a number of *Lactobacillus* species, including *Lactobacillus fermentum*, having the capacity to inhibit the growth of gram-positive bacteria, such as *Staphylococcus aureus* (ZDI: 8 – 17 mm) and *Bacillus cereus* (ZDI: 12 – 22 mm). The two *Lactobacillus fermentum* isolates from buffalo milk had ZDI of 19 mm, while the *Lactobacillus oris* conferred ZDI of 18 mm, against *Listeria monocytogenes* (Melia *et al.*, 2017). The broad spectrum antibacterial activity of *Lactobacillus fermentum* has been demonstrated earlier (Ilayajara *et al.*, 2011; Ramasamy and Suyambulingam, 2015; Podolsky, 1998), in which the LAB showed growth

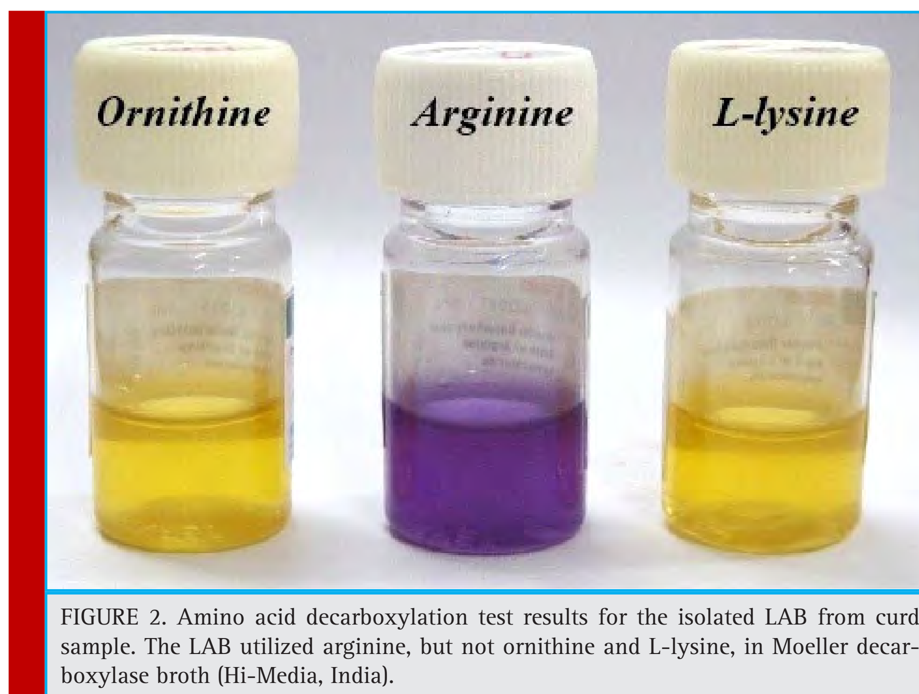

FIGURE 2. Amino acid decarboxylation test results for the isolated LAB from curd sample. The LAB utilized arginine, but not ornithine and L-lysine, in Moeller decarboxylase broth (Hi-Media, India).

inhibitory activity against gram-positive (*Staphylococcus aureus*, *Enterococcus* spp., *Streptococcus* spp., *Bacillus subtilis*) and gram-negative (*Proteus* spp., *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*) bacterial pathogens.

Kang *et al.* (2017) reported the enhancing killing of *Staphylococcus aureus* strains by *Lactobacillus salivarius* except *Staphylococcus aureus* ATCC 25923 strain, the growth of which was fully killed by *Lactobacillus fermentum.*

Sharma *et al.* (2016) isolated two lactic acid bacteria: *Pediococcus acidilactici* and *Lactobacillus casei* from milk cream and lassi, respectively, which had antibacterial activity against a large number of potential

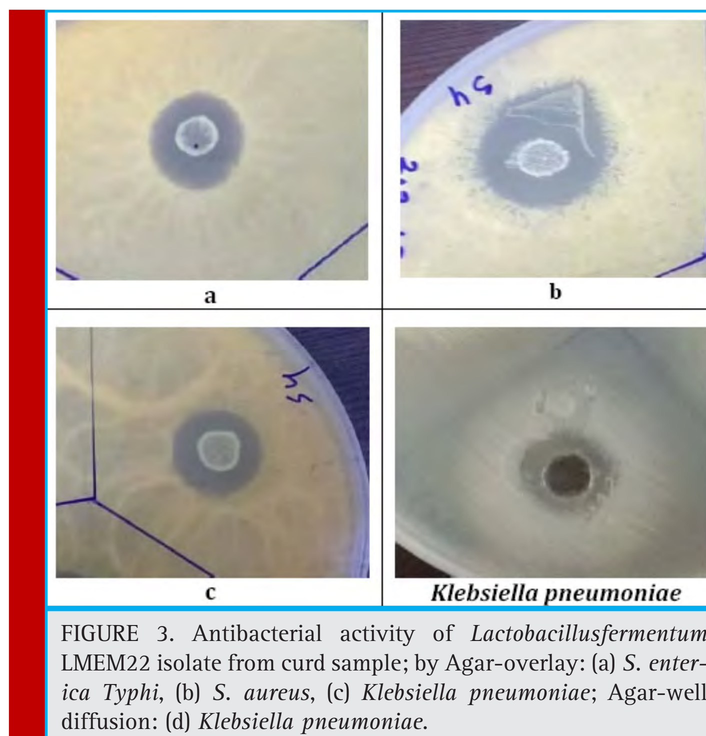

FIGURE 3. Antibacterial activity of *Lactobacillusfermentum* LMEM22 isolate from curd sample; by Agar-overlay: (a) *S. enterica Typhi*, (b) *S. aureus*, (c) *Klebsiella pneumoniae*; Agar-well diffusion: (d) *Klebsiella pneumoniae.*

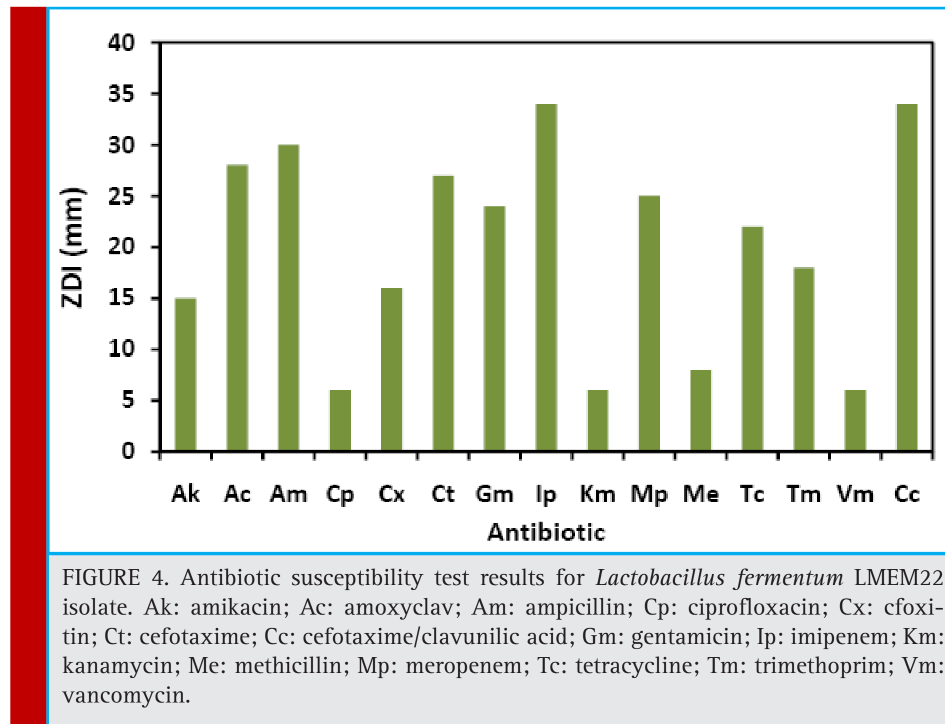

FIGURE 4. Antibiotic susceptibility test results for *Lactobacillus fermentum* LMEM22 isolate. Ak: amikacin; Ac: amoxyclav; Am: ampicillin; Cp: ciprofloxacin; Cx: cfoxitin; Ct: cefotaxime; Cc: cefotaxime/clavunilic acid; Gm: gentamicin; Ip: imipenem; Km: kanamycin; Me: methicillin; Mp: meropenem; Tc: tetracycline; Tm: trimethoprim; Vm: vancomycin.

food-borne bacteria, viz., *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Clostridium perfringens* and *Bacillus cereus* having respective ZDIs range from 12 mm to 20 mm and from 13 mm to 19 mm. In the current study, the agar-well diffusion had ZDIs of 10.67±2.08 mm (*Staphylococcus aureus*) to 12±1.00 mm (*Bacillus cereus*), for gram-positive, and 13.00±2.65 mm (*Escherichia coli*) to 18.00±3.00 mm (*Salmonella enterica* Typhi), for gram-negative bacteria (Table 2). The *Lactobacillus fermentum* LMEM 22 isolate, following agar overlay method, also had antibacterial activity against the indicator bacterial strains (Figure 3), displaying ZDIs from 17.00±1.73 mm (*Listeria monocytogenes*) to 20.00±1.00 mm (*Enterococcus faecalis*), for gram-positive, and from 18.00±2.00 mm (*Escherichia coli*) to 33±2.65 mm (*Acinetobacter baumannii*) for gram-negative bacteria (Table 3).

Table 1. Sugar fermentation test results for the isolated LAB from curd sample

Sugars	Utilization	Sugars	Utilization
Adonitol	-	D-Melezitose	+
Arabinose	W	Raffinose	+
Cellobiose	+	Rhamnose	+
Dextrose	+	D-Ribose	+
Esculin	-	Salicin	-
Glucose	+ (g)	Sorbitol	+
Lactose	+	Sucrose	+
Mannitol	+	Trehalose	+
Mannose	+	Xylose	+
Melibiose	+	D-Galactose	+

+: Strong fermentation; w: week fermentation –: No fermentation

The bacteriocin activity of the isolated LAB, *Lactobacillus fermentum* LMEM 22, has been shown in Table 2, while the Table 3 depicts the 'R' values of the isolated LAB. Iyapparaj *et al.* (2013) isolated *Lactobacillus* sp. MSU3IR strain, which against pathogenic bacteria: *Staphylococcus aureus* and *Pseudomonas aeruginosa*, displayed higher bacteriocin activity (393.2 ± 2.61 to 556.0 ± 5.34 AU/ml) in MRS medium, compared to the activity value (341.2 ± 2.36 to 473.2 ± 3.96 AU/ml) as recorded in *Lactobacillus* selection broth. Earlier, the 'R' values of curd lactobacilli strains ranged 3.00 – 13.17 mm, while the bacteriocin activity, in terms of arbitrary units (AU/ml), ranged 155.60 – 293.33, against MDR *Klebsiella pneumoniae* clinical isolates (Mandal and Halder, 2018).

In another study, the probiotic lactobacilli had excellent antibacterial activity against gram-negative human pathogenic bacteria (*Escherichia coli*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhi) displaying 'R' values ranging from 3.17 ± 0.29 to 15.33 ± 1.26 mm, and the bacteriocin activity ranging from 233.34 ± 45.54 to 280.56 ± 83.67 AU/ml (Halder *et al.*, 2017). As per the report of Shehata *et al.* (2016), among nine isolates of LAB, one (*Lactococcus lactic* subsp. *lactis*) had strong activity (1600 AU/ml)

Table 2. Agar-well diffusion test results in terms ZDI (mm) and the calculated bacteriocin activity (Au/ml) of *Lactobacillus fermentum* LMEM22 against gram-positive and gram-negative indicator bacterial strains.

Indicator strains	Bacterial isolates	ZDI range (Mean ± SD)	Bacteriocin activity
Gram-negative	*E. coli*	10-15 (13.00±2.65)	173.33
	Pr. vulgaris	12-14 (13.33±1.15)	177.73
	A. baumannii	16-20 (17.33±2.31)	231.07
	Ps. aeruginosa	15-16 (15.67±0.58)	208.93
	K. pneumoniae	14-17 (15.67±1.53)	208.93
	S. enterica Typhi	15-21 (18.00±3.00)	240.00
Gram-positive	*B. cereus*	11-13 (12.00±1.00)	160.00
	E. faecalis	10-13 (11.67±1.53)	155.60
	S. aureus	9-13 (10.67±2.08)	142.27
	L. monocytogenes	10-13 (11.67±1.53)	155.60

Au/ml: arbitrary units per milliliter

against *Klebsiella pneumoniae*, while, four isolates had bacteriocin activity of 800 AU/ml against *Escherichia coli* (for *Lactobacillus paracasei*), *Streptococcus pyogenes* (for *Lactobacillus gasseri*), *Staphylococcus aureus* (for *Lactobacillus rhamnosus*) and *Salmonella senftenberg* (for *Lactobacillus gasseri* RM28). The current investigation demonstrates the capacity of antibacterial activity of *Lactobacillus fermentum* LMEM 22 for the indicator microorganisms, consisting of both gram-positive and gram-negative pathogenic bacteria, with an overall bacteriocin activity (AU/ml) of 142.27 – 240.00 and the 'R' values of 5.5 – 13.5 mm.

The antibiotic susceptibility test results for *Lactobacillus fermentum* LMEM 22 isolate is shown in Figure 4. One of the most important probiotic features, defining safe for human consumption, of lactic acid bacteria is being their antibiotic sensitivity, and the intrinsic resistance (chromosomally conferred from point mutation) property as well (Georgieva *et al.*, 2015). As has been demonstrated by Benavides *et al.* (2016), the isolated *Lactobacillus fermentum* was sensitive to ampicillin (ZDI: 28 mm), cefuroxime (ZDI: 30 mm), tetracycline (ZDI: 24 mm) and amoxicillin/clavulanic acid (ZDI: 26 mm) and resistant to gentamycin and kanamycin, and the LAB was found inhibitory to *Escherichia coli* (ZDI: 13 mm) and *Salmonella* Typhimurium (ZDI: 12 mm). As per our earlier report the curd lactobacilli had sensitivity to majority of the test antibiotics displaying a common resistance to Vm (Halder and Mandal, 2016). In the instant case, the isolated LAB: *Lactobacillus fermentum* LMEM 22 showed resistance to Ak, Cp, Km, Me and Vm (ZDI: ≤15 mm; range: 6 – 15 mm), and such resistances are intrinsic as well as non-transferable (Bamidele *et al.*, 2017; Imperial and Ibana, 2016).

The *Lactobacillus fermentum* LMEM 22 was sensitive to Ac, Am, Cc, Cx, Gm, Im, Mp and Tc (ZDI: ≥21 mm;

Table 3. Agar overlay test results in terms ZDI (mm) and the calculated 'R' values for *Lactobacillus fermentum* LMEM22 against gram-positive and gram-negative indicator bacterial strains

Indicator strains	Bacterial isolates	ZDI range (Mean ± SD)	'R' value (mm)
Gram-negative	*E. coli*	16-20 (18.00±2.00)	6.00
	Pr. vulgaris	21-26 (23.33±2.52)	8.67
	A. baumannii	31-36 (33.00±2.65)	13.50
	Ps. aeruginosa	23-28 (26.00±2.65)	10.00
	K. pneumoniae	20-25 (22.67±2.52)	8.34
	S. enterica Typhi	20-25 (22.00±2.65)	8.00
Gram-positive	*B. cereus*	16-19 (17.67±1.53)	5.84
	E. faecalis	19-21 (20.00±1.00)	7.00
	S. aureus	18-20 (18.67±1.56)	6.34
	L. monocytogenes	15-18 (17.00±1.73)	5.50

SD: standard deviation; ZDI: zone diameter of inhibition.

range: 22 – 34 mm), and intermediately susceptible to Cx and Tm with ZDIs of 16 and 18 mm (ZDI criteria range: 16 – 20 mm); the LAB, while, showed resistance (ZDI: ≤15 mm) to Ak, Cp, Km, Me and Vm. Thus, the isolated LAB, in this study, has been found to be safe, on the basis of lack of transferable antibiotic resistance property (Ammor *et al.*, 2008; Imperial and Ibana, 2016), and this LAB might be useful in single-strain based probiotic formulation benefiting a large number of local population, in this part of the globe. It has been reported that multi-strain/multi-general probiotics might exhibit limited functional property for universal usage, requiring probiotics alternatives development and/or personalized probiotic approaches (Zmora *et al.*, 2018; Suez *et al.*, 2018).

CONCLUSION

The *Lactobacillus fermentum* LMEM22, which was isolated from locally available commercial curd, showed antagonistic activity against gram-positive as well as gram-negative pathogenic bacteria, with overall bacteriocin activity (AU/ml) of 142.27 – 240.00, and the 'R' value of 5.5 – 13.5, and (based upon the report available in literatures, too) there is no risk of transferable antibiotic resistance in the LAB. Thus, the isolated LAB might be useful as broad spectrum antibacterial biotherapeutics, and such native LAB isolate might be consumed alone, in place of antibiotic therapy, or can be used (based upon the antibiogram of the native LAB) in probiotic-antibiotic combination therapy. However, further studies are needed to validate the probiotic attributes of the isolated LAB, including its molecular identity as well as the antibiotic resistance management.

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Effect of pelvic floor exercise on non-specific lower back pain in post-partum women

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ABSTRACT

The present aim of the study was to investigate improvement in pain intensity and measure disability of women with constant postpartum lower back pain after and tailored exercise protocol. Herein, 30 women aged between 30-35 years having lumbo-pelvic pain after delivery of three years were included and were received tailored exercises. The subjects were classified acoording to Demographic and Anthropometric characteristics. Manual Screening for pain was done through VAS (visual analog scale) and Oswestry disability questionnaire. Group A and Group B were classified as according to the exercise protocols. Group A were subjected to pelvic floor exercise along with abdominal muscle strengthening while Group B were subjected to Spinal hyperextensions along with abdominal muscle strengthening. The results depicted that the pelvic floor exercise in combination abdominal exercise with routine treatment for back pain provide significant benefits in terms of pain relief and disability over routine treatment as compared to spinal hyperextensions along with abdominal muscle strengthening. A correlation was also established between the changes in disability and pain intensity in between two groups.

KEY WORDS: PELVIC FLOOR EXERCISE, SPINAL HYPEREXTENSIONS, POST PARTUM WOMEN, LOWER BACK PAIN

INTRODUCTION

Back pain is common phenomenon in female which is experienced during postpartum and is expected to prolong for 4-6 months affecting activities of daily living. The majority of women recover from pregnancy related mechanical lumbo-pelvic pain within 6 months of delivery However, studies show that 25 % of females still have persistent non-specific lower back pain 2-3 years after delivery and which interferes with their daily activities (Tersi et al., 2015, Corso et al., 2016 and Gausel et al., 2016).

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The pelvic floor is the area underneath the pelvis which consists of muscles and connective tissues a complex structure. It provides support to the abdominal viscera including uterus, bladder and other viscera. The core muscles include pelvic floor muscles, transverse abdominis, multifidus, internal and external oblique, rectus, abdominus, erector spinae, quadratus lumborum, lattismus dorsi, and gluteus maximus. The muscles involved in lower back pain are erector spinae, oblique muscles. Since the core muscles and back muscles are involved into spinal rotation, so they are related to each other, (Javadian et al., 2015, Chevidikunnan et al., 2016 and Teymuri et al., 2018).

The lower back is related to multiple factors which recount dynamic instability of pelvis and hormonal changes this joint instability initiate the deep muscle activation which demonstrates the sacroiliac stiffness, based on the earlier studies guided exercises for pelvic floor and abdominal are recommended. Consequently, the design of the study should be suitable with observation of relation between motor control and reduction in symptoms. Only few studies describe tailored exercises therapy for strengthening pelvic floor muscles and spinal stability and methods adapted are kegel's exercise protocol, spinal extension and abdominal strengthening. The intervention adapted should target the outcomes throughout the whole intervention period (Portney et al., 2014, Tondel et al., 2016 and Bhadauria et al., 2017).

The exercises protocol adapted to activated the group of deep and superficial muscles which results in improvement in the ODQ and pain score also demonstrating core strengthening exercise of conventional exercises (Saragiotto et al., 2016, Ferla et al., in 2016), also improving on the biomechanics (Bi et al., 2013) also some author established more emphasis should be given over the exercise during the third trimester of pregnancy where chances of weight increase are more established. (Domenjoz, 2014 & Kolu, 2014). Also pelvic floor exercise for organ prolapse and urinary incontinence plays an important role in improving the symptoms and to treat musculoskeletal and movement impairments in women (Kurz et al., 2017). The weakness of pelvic floor muscles and relationship of urinary incontinence was established in both men and women and importance of screening of back pain in patients with urinary incontinence (Cassidy et al., 2017)

The aim of the present study was to investigate weekly improvement in pain intensity and measure disability of women with constant postpartum lower back pain after and tailored exercise protocol. A secondary aim was to establish correlation between the changes in disability and pain intensity in between two groups.

MATERIALS AND METHODS

30 women after lumbo-pelvic pain after delivery of three years were included in the study and received tailored exercises. The subjects aged between 30-35 years were included in the study. The study was performed at RLJT Hospital & Research Centre, Jhunjhunu. Exclusion criteria BMI > 25, traumatic backache history of neurological or autoimmune disorder, history of Pelvic organ prolapse, respiratory or metabolic disease The experiment was conducted on the basis of Demographic data and Anthropometric characteristics of the subjects was recorded including Name, Age, Gender, Height and Weight, therefore BMI was also recorded prior to the study. Manual Screening for pain was done through VAS (visual analog scale) and Oswestry disability questionnaire.

Visual analogue scale (VAS) - the pain scale was used to measure the degree of pain, after presenting the pain levels on a straight line of 10 cm without gradation. The calculations were done as 0 presenting as no pain and 10 presenting as extreme unbearable pain. The marking were made from 0-10 in centimeters, (Kersten et al., 2014).

Oswestry Low Back Pain Disability Questionnaire-

The instrument was developed to illustrate functional disability and to measure the clinical reduction and improvement lower back pain. It a self questionnaire to be filled by the patient based on the disability faced during activities of daily living, the questionnaire includes 10 questions related to pain management, personal care, walking, sitting, standing, sleeping, and social life with a scoring from 0-5 for every question with a total scoring of 50.Thirty women with non specific chronic LBP were recruited and randomly assigned into two groups, an experimental group A (n=15) and experimental group B (n=15). The assessment was performed in crook lying position, Pre and post data was collected before intervention, pain intensity was measured on Visual Analogue Scale (VAS) and functional disability was assessed using Oswestry Disability Questionnaire (Pereira et al., 2017).

Total protocol was performed for 8 weeks

Group A: Pelvic floor exercises:

Women in this group were explained the importance of exercises, anatomy of pelvic floor exercises. The patients were taught to contract their pelvic floor muscles and to squeeze with maximum applied effort and hold for 3-4 seconds without holding the breath.

1st phase: The patients were asked to complete 15-20 repetition in sitting and lying for 2-3 sets in a day for first 3 weeks in crook lying positions

2nd phase: For 4th -6th week onwards the repetition were increase nearly double and as per the comfort of the patient completing 2-3 times in a day, patient were advice to practice the session in two positions i.e. sitting and lying

3rd phase: 7th week onwards the patient was asked to increase the no. of repetition to 40-50 with same no. of sets in a day and continued till end of 8th week, patient was advice to continue the exercises in lying , sitting and standing

Abdominal exercises:

A. Pelvic tilt:

- Subject lies in supine lying position with knees flexed, arm placed on the side.
- Subjects were push the lower trunk into the floor and contract the abdominal and glueteal muscles, with rotating the pelvis upwards and inward and making a bridge back off from the floor , the same position is holded for 5 seconds each and relax period of 5 seconds repeating 10 times for 2- 3 times in day.

B. Partial curls:
Subjects is asked to lie down supine with knees flexed, arms are extended the subject is asked to rest hands over the legs and then sliding the fingers over the knees with flexion of the trunk and slightly lifting the shoulders, exercises should be repeated 10 times with 2-3 sets in a day .Subjects were instructed not to hold breath in any exercises.

C. Diagonal curls:

Subject in supine lying position with arm in forwards position , subject is asked to lift the shoulder off the floor bringing the left shoulder towards the right knee and vice-versa then repetition for 10 times for 2-3 times in a day

Group B:

Women in this group are trained for back extensor exercise along with abdominal muscle strengthening.

Spinal hyperextensions: Subjects were asked to lie down in prone position with hand on side, keeping weight on the hands were asked to lift and extend the spine and hold for 5-10 second repeating the process for 10 times and 2-3 times in a day.

Prone on elbows: Subjects were asked to lie down in prone position with elbow on side, keeping weight on the elbow were asked to lift and extend the spine and hold for 5-10 second repeating the process for 10 times and 2-3 times in a day.

Abdominal exercises: Isometrics abdominals: Subjects were asked to lie down supine with knee flexed and hands under the lower back, they were told to contract the anterior abdominal muscles and pres the hand with the back, holding the position for 5-10 second and repeating the same exercise at least 10 times for 2-3 sets in a day during the exercises the breath holding should be avoided followed by same abdominal exercise taught to group A

RESULTS AND DISCUSSION

Data analysis: The data analysis was done using Statistical Package for Social Sciences software Version 19 applying the descriptive data included mean standard deviation was also calculated with paired t test and ANOVAs test were applied to calculate the difference between pre and post treatment intra groups. The data was analyzed using with t-value in inter group and in intra group using F distribution value and the F-crit value. The result will be significant at $p < .05$.and if $p>0.05$ considering it non- significant.

Subjects (30) were included in the study with mean age of 33.77 ±1.44 in Group A and 32±1.46 in Group B years. The mean height calculated was 164±7.89 and cm and 156.44±7.59 cm, mean weight was 62±9.50 and 60.5±8.54 in kg, The mean BMI was also calculated as 23±4.58 and 25.73±2.49 respectively for group A and B.

As shown in table 2 and 3 there is significant effect on the VAS score of the patient at baseline in both the experimental Group A & B, there was an significant different seen in the score in experimental group A, but there was no significant improvement seen in the ODQ in group B

As explained in table 4 the difference within the group for VAS score that the mean square between treatments, 2.7 not much larger is than the mean square within treatments, 1.02381. The F value for VAS i.e. MSB/MSW after the treatment is 2.63. The values showed not much variability between treatment groups and within treatment groups. The smaller the ration less confident the score are

Table 1. Illustrates the demographic data and anthropometric data of the subjects

Characteristics	Group A	Group B
Age (Mean ± SD)	33.77±1.44	32±1.46
Height (In cm) (Mean ± SD)	164±7.89	154.44±5.06
Weight (In kg) (Mean ± SD)	64±8.14	60.5±8.54
BMI (Mean ± SD)	23±4.58	25.73±2.49

Table 2. Illustrates Group A-Experimental group (VAS and Oswestry Disability Questionnaire)				
	MEAN	SS/df	T VALUE	P VALUE
Visual Analog scale (VAS)	-3.07	0.78	8.829	< 0.00001
Oswestry Disability Questionnaire (ODQ)	-26.13	34.55	10.508	< 0.00001

Table 3. Illustrates Group B-Experimental group (VAS and Oswestry Disability Questionnaire)				
	MEAN	SS/df	T VALUE	P VALUE
Visual Analog scale (VAS)	-3.73	0.92	9.427	< 0.00001
Oswestry Disability Questionnaire (ODQ)	-2.07	14.50	0.53036	<0.3002

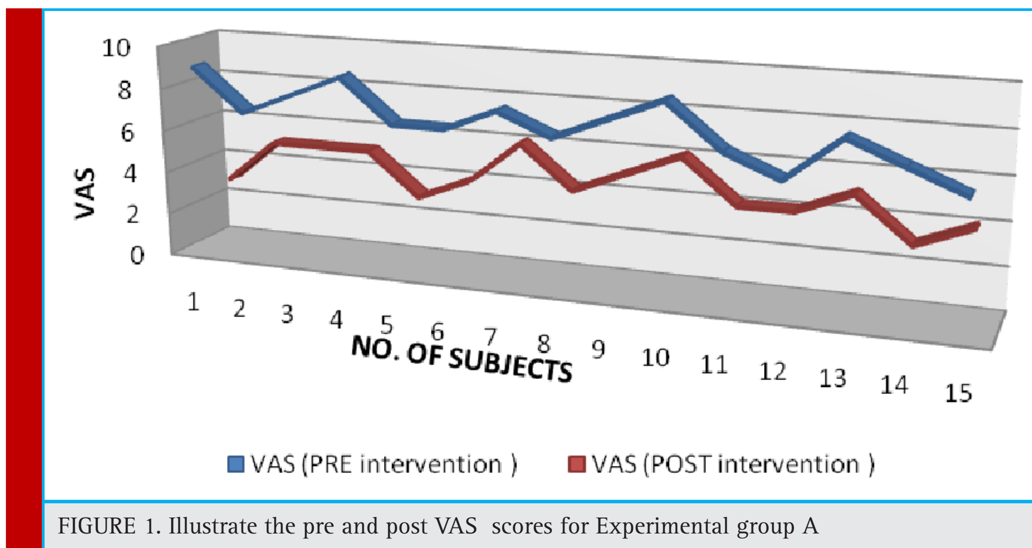

FIGURE 1. Illustrate the pre and post VAS scores for Experimental group A

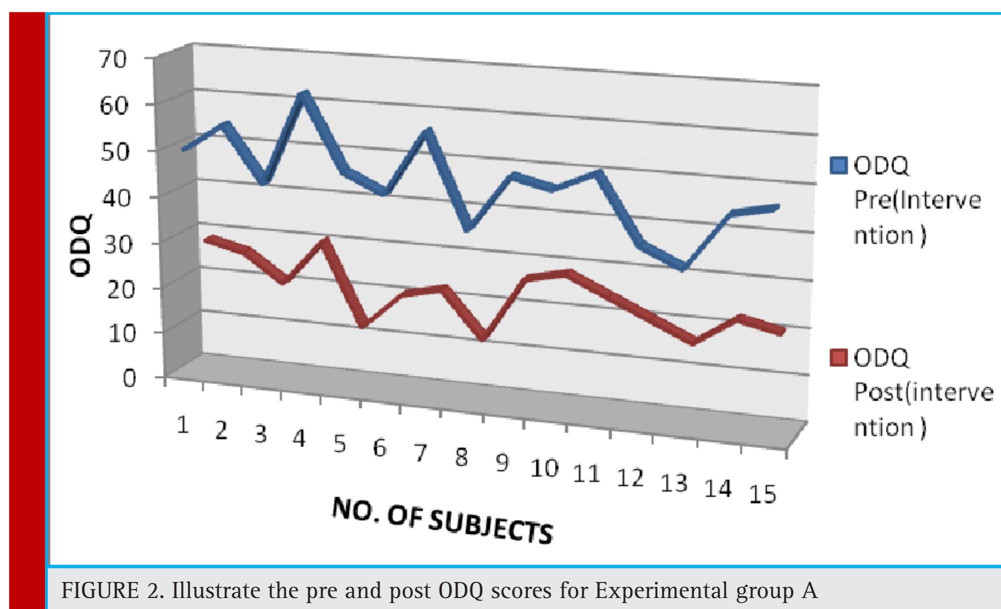

FIGURE 2. Illustrate the pre and post ODQ scores for Experimental group A

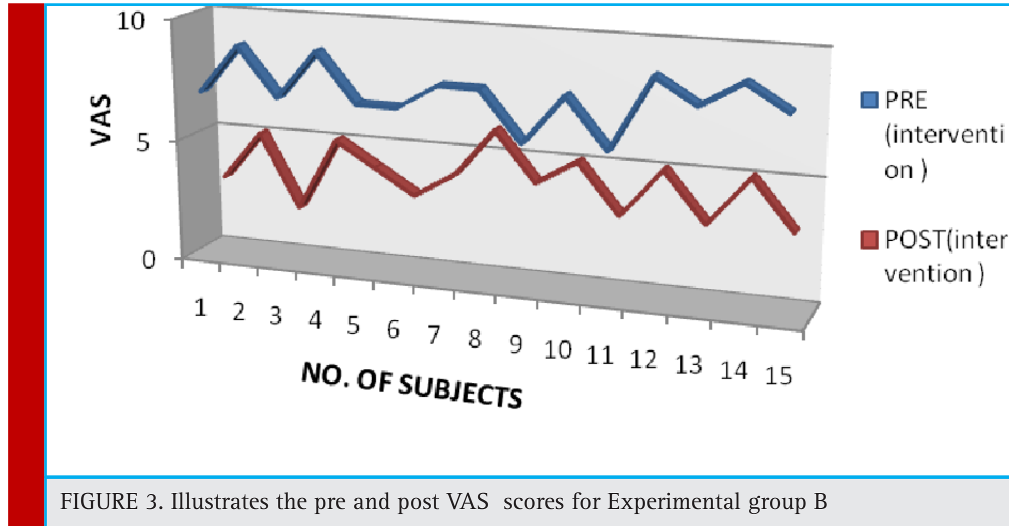

FIGURE 3. Illustrates the pre and post VAS scores for Experimental group B

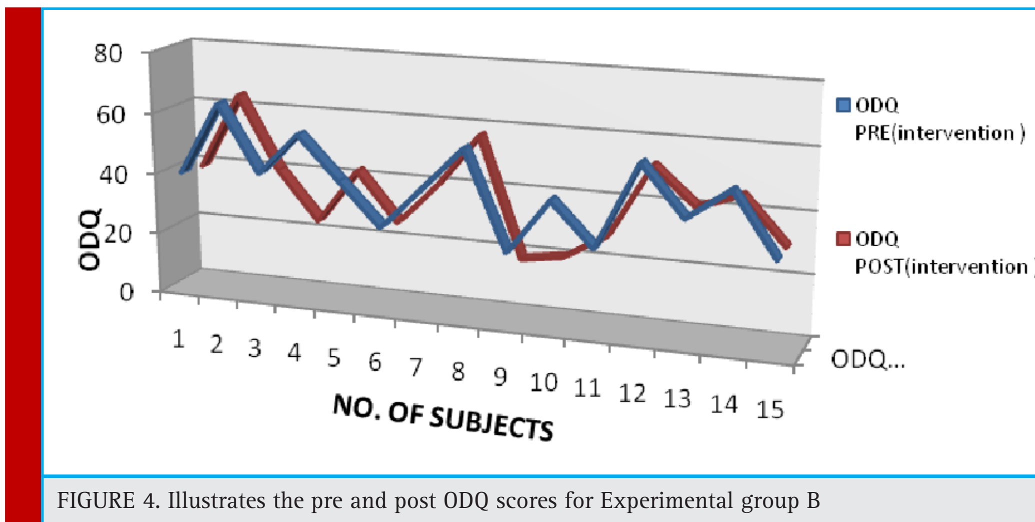

FIGURE 4. Illustrates the pre and post ODQ scores for Experimental group B

Table 4. The above two table illustrate the comparison between groups and within groups

Source of Variation for VAS	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.7	1	2.7	2.637209	0.115593	4.195972
Within Groups	28.66667	28	1.02381			
Total	31.36667	29				
Source of Variation for ODQ	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3349.633	1	3349.633	54.9206	4.53E-08	4.195972
Within Groups	1707.733	28	60.99048			
Total	5057.367	29				

rejecting the H1 and accepting the H0 hypothesis ,stating there was no difference in VAS score in between two group A and B showing similar results. Secondly the P value is more than the significant the p-value is 0.115593. The result is not significant at $p < .05$. The difference within the group for ODQ score that the mean square between treatments 3349.633, much larger is than the mean square 60.99048 within treatments The F value for ODQ i.e. MSB/MSW after the treatment is 54.9206. As the value shows much variability between treatment groups and within treatment groups. The larger the ratio more is the confidence of score in rejecting the H0 and accepting the H1 hypothesis, stating there was a difference in ODQ score in between two group A and B. The f-ratio value is 54.9206. The p-value is $< .00001$. The result is significant at $p < .05$.

We hereby discuss that pelvic floor muscles make up a large part of the body's core, which is the foundation for all movement, balance, stability and flexibility. The pelvic floor muscle exercise protocol used in the present study was designed to co-activate superficial, deep core muscles and abdominal exercises which resulted in significantly better improvements in pain scores and ODQ score. Javadian et al., (2015) compared with routine treatment with back extension exercise and abdominal exercises. Back function parameters were improved over 8 weeks regular exercise protocol from baseline in group A, with significant between-group differences In the previous studies VAS and Oswerty disability index was used to demonstrate the effect of pelvic floor exercises, (Mohammad et al., 2011 & Teymuri et al., 2018).

These results are consistent with the findings of others, who demonstrated the superiority of a pelvic floor strengthening protocol to over spinal extension exercises for LBP. Others have suggested, however, that pelvic floor exercises do provide benefit over conventional physiotherapy exercises in terms of pain or disability reduction (Bi et al., 2013 & Bhadauria et al., 2017).

A spinal extension exercises protocol comprising exercises every day for 8 weeks was found to provide no additional significant improvement in ODQ score compared with routine treatment. The more intense training and longer treatment period (a minimum of 15 contraction cycles/day for 12 weeks) may explain the significant improvements in pain and disability seen in the present study. Improving the biomechanical characteristics of muscles in patients with LBP may require longer duration in case of patients treated with spinal extension exercises

The current studies also have some limitations. The sample size was too small to draw any firm conclusions. Finally, as the study population included patients with nonspecific low back pain, it is not possible to generalize these findings to those with low back pain of specific cause.

CONCLUSION

In conclusion, the PFM exercises in combination abdominal exercise with routine treatment for back pain provide significant benefits in terms of pain relief and disability over routine treatment. Also it may be recommended that PFM exercises should be an routine protocol for any mechanical lumbo-pelvic pains. Further, larger-scale studies with long-term follow-up are required before these findings can be applied to a wider patient group.

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Phylogenetic analysis of target of rapamycin (TOR) kinase gene of some selected plants species

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ABSTRACT

TOR kinase has been reported to regulate number of biological processes, including central dogma, which collectively contributes to cell growth in all the organisms. The major role of the target of rapamycin (TOR) kinase is to encourage cell growth in response to favorable conditions. Up to some extent,nucleotide and amino acidsequencesof TOR kinase were found to be similar in all the organismswherein it has been identified or characterized.In order to assess the phylogenetic relationship and conservative nature of TOR gene among 32 different plants species TOR gene sequences and protein sequence retrieve from public repository database NCBI.Sequence length and GC% of each sequence were determined. Maximum GC% was found in TOR kinase gene of *Brachypodium distachyon* (46.18%). All the 32 TOR gene sequences contained more than 43% of GC. Phylogenetic tree constructed using Neighbor-joining method separated TOR kinase into two distinct groups *i.e.* monocots and dicots. Sequences of TOR kinase from similar family of plants were grouped together signifyingits conserved nature within the family. The phylogenetic tree of TOR gene at both nucleotides and proteins level from different species perfectly reflects phylogenetic relationships of the species. This strong conservation of *tor* genes among all the species including in this investigation advocate the general significance of this kinase and, consequently, the entire TOR pathway.

KEY WORDS: CELL GROWTH; NEIGHBOR-JOINING; PHYLOGENETIC TREE; *TOR* KINASE

INTRODUCTION

The modulation of growth rate in a particular environmental condition such as nutrient availabilityis necessary for continued existence. Plant growth is largely dependent on surrounding environmental information. It includes cell growth coupled with cell proliferation and cell expansion depends onexogenous factors such as stresses and nutrient availability. Unlike animals, in plants, postembryonic growth is directly influenced

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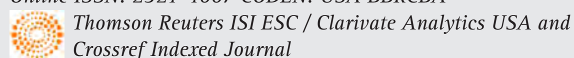

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by availability of nutrients and energy source that are present outwardly or generated by various cellular processes (Nanjareddy et al. 2016). However, so far, very few secrets have been revealed on mechanism that how this information is perceived and transduce into coherent growth and developmental decisions.One of the most important pathways that are found in all eukaryotes is the one related to the target of rapamycin (TOR) protein kinase.

The target of rapamycin (TOR), a Ser/Thr protein kinase, has emerged as a key player of nutrient, energy, and stress signaling networks (Dobrenel et al. 2013; Yuan et al. 2013). It is a large protein that belongs to the phosphoinositide 3-kinase-related kinase family and is highly conserved among all eukaryotes (Robaglia et al. 2012). Numerous components of the TOR signalingmachinery have beenidentified in model plant *Arabidopsis*. Various members of the TOR complex such as the RAPTOR1/RAPTOR2, LST8-1/LST8-2, S6K1/S6K2, ribosome protein small subunit6 (RPS6A/B), type 2A-phosphatase-associated protein 46 kD (TAP46), and ErbB-3 epidermal growth factor receptor binding protein have been reported in photosynthetic eukaryotes through sequence homology searches from *C. reinhardtii* to Arabidopsis plants (Creff et al. 2010; Ahn et al. 2011; Moreau et al. 2012; Ren et al. 2012; Xiong and Sheen, 2012).

In this era of high throughput gene and genome sequencing, prediction of function of a gene is a key step. Various reverse genetics techniques like site directed mutagenesis and RNAi are effective to solve this purpose. Another easier way to predict function of a particular gene and phylogenetic relation between different species iswith the use of bioinformatics. Nowadays, increasing sequenced genomesof diverse plants are providing new opportunities to study gene families in an evolutionary context. Based on these facts, present investigation was conducted to evaluate the phylogenetic relationship and sequence similarity of TOR kinase genes from different plants species and make an efforts to know the conserve nature of TOR gene at both nucleotide and protein sequence level. Phylogenetic tree analysis on basis of conserved nature and similarity of known sequence helps us to predict the function of a gene and also exhibit the phylogenetic relationships of the species (John et al. 2011). However, for a deeper understanding of the gene function, it is helpful to go beyond cataloguing of similarities and differences and to understand how and even why these similarities and differences arise.

MATERIAL AND METHODS

Nucleotide sequence of TOR kinase gene of *Zea mays* was retrieved from NCBI database for its further use as bait sequence to isolate other sequences of different plant species using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/). TOR gene nucleotide sequences of different plants were selected on the basis of E-value (within 0 to 1e – 50). DNA sequences were aligned with ClustalW (Thompson et al. 1994) and alignments were subsequently adjusted manually using BioEdit (Hall, 1999). Sequence length and GC% of each sequencewas calculated by MEGA6 (Tamura et al. 2011). Protein sequences were also deduced from all retrieved sequences of TOR kinase genes and were aligned using ClustalW and go for construction of phylogenetic tree.

To evaluate the genetic relationship between retrieved nucleotide sequences of TOR kinase gene, a phylogenetic tree was constructed using Neighbor-joining (NJ) method. The output data was processed using MEGA 6 to draw the phylogenetic tree. The bootstrap consensus tree (Felsenstein, 1985) inferred from 1000 replicates was selected to represent the evolutionary history of the 32 TOR kinase genes under study. Phylogenetic tree was also constructed for amino acid sequences of TOR kinase genes two know the sequence homology at protein level.

RESULTS AND DISCUSSION

In this study, TOR kinase gene sequences of 32 plants species belongs to 11 familieswere selected and retrieved from GenBank for phylogenetic analysis. The sequence lengths of all the TOR genes with GC% are given in Table 1. Maximum GC% was found in TOR kinase gene of *Brachypodium distachyon* (46.18%). All the 32 TOR gene sequences containedmore than 43% of GC.

The evolutionary relationships between the plants were evaluated by phylogenetic analysis of the aligned nucleotides and amino acids sequence of their TOR kinase gene. TOR kinase gene sequences currently available in the database are either full length mRNA sequences or predicted sequences obtained from annotation of genome. The phylogenetic tree obtained by Neighbor-joining method showed two distinctphylo-groups of TOR kinase genes from monocots and dicots (Fig 1). Group I containing only monocots included *Zea mays, Setaria italica, Sorghum bicolor, Brachypodium distachyon, Oryza sativa, Oryza brachyantha, Phoenix dactylifera, Elaeis guineensis* and *Musa acuminate*. Cluster of monocots further divided in two subgroups, wherein subgroup I-acontained three gene sequences, two from family Arecaceae and one from family Musaceae. The plant species *Elaesis guineensis* and *Phoenixdactylifera* belongs to same family Arecaceae and also exhibited high degree of similarity than plant species *Musaacuminata* belongs to family Musaceae in subgroup I-a.

This result also revealed that the relatedness of these two families were higher than the other family included in the present studies. While, subgroup I-b comprised 7

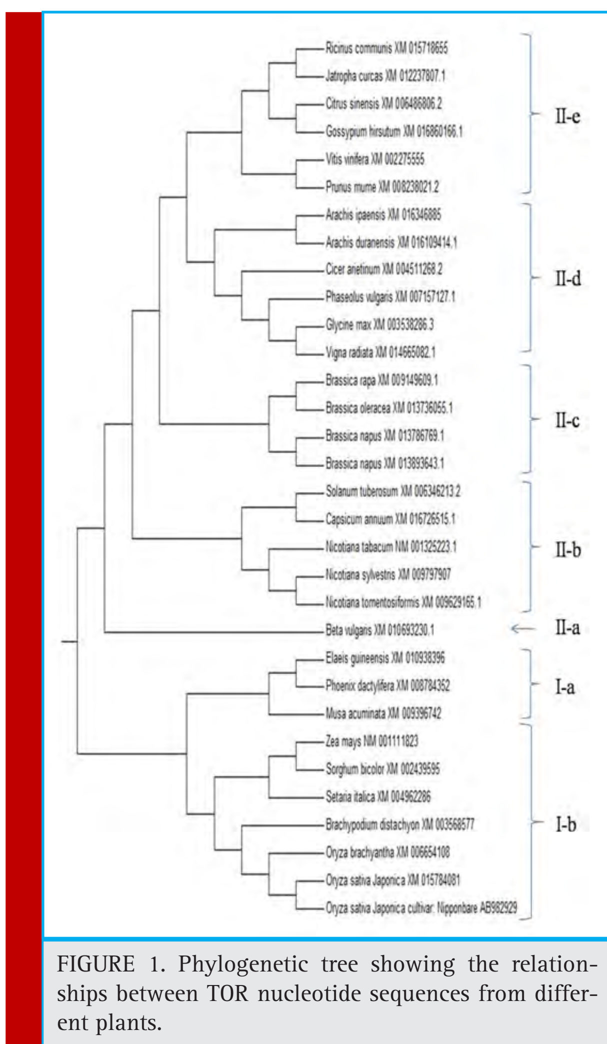

FIGURE 1. Phylogenetic tree showing the relationships between TOR nucleotide sequences from different plants.

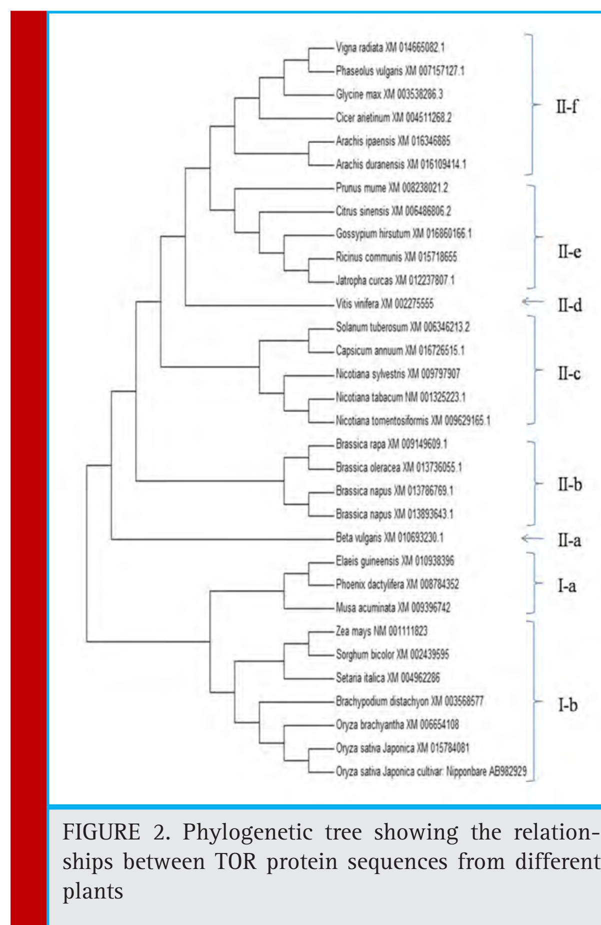

FIGURE 2. Phylogenetic tree showing the relationships between TOR protein sequences from different plants

sequences,all belongs to Poaceae family. In this group the plant species of genus *Oryza* is closely related with *Bracypodiumdistachyon* while *Zeamay s*exhibited higher similarity with plant species *Sorghum bicolor* and *Setaria italica*. In group II, all the sequences of TOR kinase gene of dicot plants clustered into 5 subgroups. TOR gene sequence of *Beta vulgaris* was lone species in subgroup II-a whereas, remaining 4 subgroups clustered according to their family such as subgroup II-b consist of 5 species of Solanaceae, Subgroup II-cwas occupied bythe 4 species of Brasicaceae while 6 species of Fabaceae family gathered in subgroup II-d. On the other hand, five families namely Euphorbiacea, Malvaceae, Rootaceae, Rosaceae and Vitaceae grouped into subgroupII-e.

Inphylogenetic tree of amino acid sequences of TOR kinase divided into two groups of monocots and dicots (Fig 2) with a similar clustering pattern to nucleotide sequenc eexcept *Vitis vinifera* which formed a separate group II-d in phylogenetic tree of protein sequences. In group I of monocots, two subgroups of 7 and 3 plants were formed. In group II of dicots, six subgroups were formed. *Vitis vinifera* and *Beta vulgaris* fell into two separate groups whereas, other groups contained 4,5 and 6 species of Brasicaceae, Solanaceae and Fabaceae respectively whereas, 5 genera clustered inII-e. Above mentioned results showed the highly conserved nature of TOR gene among different plant species at family level. The results obtained are in accordance with previous research finding reported by John et al. (2011) as they observed similar pattern of clustering during phylogenetic analysis of TOR proteins of different species included animal kingdom, fungi, algae and higher plants. In higher plants all the studied plants species are separated into two cluster i.e. monocots and dicots. The plants species *Oryzasativa*, *Sorghum bicolor and Zea mays* clustered together exhibited the similarity of results obtained during present studies. The amino acid sequences were found to be conserved among a wide variety of plants including all the cereals andin many dicots. Our results are also supported by another investigation carried out by Dobrenel et al. (2011) in which protein sequences of TOR kinase from plants, animals, yeasts, algae and moss were aligned and monocots and dicotsgrouped in separate clusters. The plants species

Table 1. List of source plants of *tor* genes utilized in investigation and their features

Plant	Accession number	Family	Size (bp)	GC%
Zea mays	NM_001111823	Poaceae	7691	44.80
Nicotiana sylvestris	XM_009797907	Solanaceae	6552	43.86
Setaria italica	XM_004962286	Poaceae	7899	45.80
Sorghum bicolor	XM_002439595	Poaceae	7544	44.55
Brachypodium distachyon	XM_003568577	Poaceae	8263	46.18
Oryza sativa	XM_015784081	Poaceae	7659	45.05
Oryza brachyantha	XM_006654108	Poaceae	7708	44.75
Oryza sativa	AB982929	Poaceae	7398	44.59
Elaeisguineensis	XM_010938396	Arecaceae	7722	44.28
Musa acuminata	XM_009396742	Musaceae	7722	44.51
Vitisvinifera	XM_002275555	Vitaceae	7791	44.15
Phoenix dactylifera	XM_008784352	Arecaceae	3530	43.63
Ricinuscommunis	XM_015718655	Euphorbiaceae	7993	43.69
Arachisipaensis	XM_016346885	Fabaceae	8105	44.45
Nicotiana tabacum	NM_001325223.1	Solanaceae	7488	44.78
Solanum tuberosum	XM_006346213.2	Solanaceae	7929	43.93
Capsicum annuum	XM_016726515.1	Solanaceae	7581	44.00
Citrus sinensis	XM_006486806.2	Rutaceae	7929	43.65
Nicotiana tomentosiformis	XM_009629165.1	Solanaceae	6982	43.81
Jatropha curcas	XM_012237807.1	Euphorbiaceae	6910	43.14
Gossypiumhirsutum	XM_016860166.1	Malvaceae	7904	44.00
Arachisduranensis	XM_016109414.1	Fabaceae	8144	44.51
Prunusmume	XM_008238021.2	Rosaceae	7991	44.50
Glycine max	XM_003538286.3	Fabaceae	8287	42.97
Vigna radiata	XM_014665082.1	Fabaceae	8372	43.11
Cicer arietinum	XM_004511268.2	Fabaceae	8411	42.42
Beta vulgaris	XM_010693230.1	Amaranthaceae	8031	43.71
Brassica rapa	XM_009149609.1	Brassicaceae	7800	44.44
Brassica napus	XM_013786769.1	Brassicaceae	7581	44.60
Brassica napus	XM_013893643.1	Brassicaceae	7880	44.37
Brassica oleracea	XM_013736055.1	Brassicaceae	7630	44.39
Phaseolus vulgaris	XM_007157127.1	Fabaceae	4186	43.17

Oryza sativa subsp. Japonica, *Oryza sativa* subsp. Indica, *Bracypodiumdistachyon*, *Sorghum bicolor and Zea Mays*are grouped together and formed the separate cluster of monocot species which showed similarity with results obtained during present investigation.

During phylogenetic analysis and sequence similarity search of wheat TOR gene with other plant species Sapre et al. (2016) observed the same clustering pattern of plant species as obtained in this investigation. The results of this investigation are not contradicted the results obtained by Nanjareddy et al. (2016) during the phylogenetic analysis of bean TOR gene which confirmed that this gene belongs to the legume group and is closely related to the *G. max* and *M. truncatula* TOR genes which showed the highly conserved nature of TOR gene at family level. In our studies all the plants species including in present investigation are clustered together according to their family showed the similar nature of gene as mentioned by Nanjareddy et al. (2016).

CONCLUSION

During present investigation phylogenetic analysis was carried out separately among TOR kinase gene sequences and amino acid sequences of 32 plants retrieve from public repository database NCBI. The phylogenetic tree of

TOR gene and its homolog's from several plants including both monocots and dicots revealed a close relationship between plant species.All sequences were grouped separately in two groups i.e. monocots and dicots. Further, sequences from similar family grouped together perfectly reflect the conserve nature of TOR kinase gene at nucleotide and protein sequence level.This strong conservation of TOR geneamong all the studied plant speciesadvocates the general significance of this kinase and, consequently,the entire TOR pathway.

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Studies on days to calli appearance in Ethiopian mustard, *Brassica carinata*

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ABSTRACT

The effects of seven genotypes and their cross combinations, two basal media *i.e.*, B5 and MS media, two different sucrose concentrations *i.e.*, 3% and 4% sucrose and three combinations of hormones *viz*. HM_1, HM_2 and HM_3 and their interactions on days to calli appearance in *Brassica carinata* were analyzed by using CPCS software. Analysis of variance revealed that, out of four factors, only genotypes had significant effect on days to calli appearance. Nine out of eleven interactions *viz*., genotypes x hormones, genotypes x media, hormones x media, genotypes x hormones x media, hormones x sucrose, genotypes x hormones x sucrose, genotypes x media x sucrose, hormones x media x sucrose and genotypes x hormones x media x sucrose showed significant effect on days to calli appearance.

KEY WORDS: B_5, DAYS TO CALLI APPEARANCE, HORMONES, MS, SUCROSE

INTRODUCTION

Oilseed crops are the backbone of Indian agricultural economy and occupy an important position in daily diet, being a rich source of fats and vitamins. India is the second largest rapeseed-mustard growing country and accounts for 21.7% area in the world after China. Among oilseeds, rapeseed-mustard is the second most important oilseed crop of the country after groundnut and plays a significant role in Indian oil economy by contributing about 28.6% to the total oilseed production (Shekhawat *et al*., 2014). Rapeseed-mustard is the third important oilseed crop in the world after soybean (*Glycine max*) and palm (*Elaeis guineensis* Jacq.). The crop occupies an area of 33.58 million ha with a total annual production of 67.76 million tonnes and productivity 2018 kg/ha. In production, India ranks third after China (22.9%) and Canada (19.7%). The global production of rapeseed-mustard oil is around 12-14 million tonnes. In India, the crop occupies an area of 6.50 million ha with a total production of 8.02 million tonnes and productivity of 1262 kg/ha (Priyamedha *et al*., 2017).

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Over the last decades, researchers have made great efforts in developing biotechnology methods to facilitate the breeding of *Brassicas*. Research studies indicated that the modern biotechnology will have a major impact in two areas. Firstly, it provides a new range of techniques enabling the efficient selection of favourable variants in plant breeding programmes. Secondly, it provides the opportunity to improve germplasm by increasing its diversity beyond conventional genetic limitations. Due to the relative ease of genetic transformation, *Brassica* oilseed crops have been amongst the first subject to study the full range of modern biotechnology methods (Abraha *et al.*, 2008).

Conventional methods for breeding crop plants require more than six to seven years of continuous efforts to get true breeding lines after following hybridization approach, a time consuming process (Morrison and Evans, 1988). Hence, biotechnological tools including anther culture, hold a great promise in accelerating the pace of breeding programme (Guha and Maheshwari, 1964). In vitro technique of anther culture helps to achieve homozygosity very quickly (Snape, 1989). Anther culture of potential F_1 generation genotypes can be used to facilitate regeneration of stable recombinant inbreds in one to two years thereby saving time and resources for their further use directly as commercial cultivars and/or in structural and functional genomics. The object of this study was to investigate the response of different genotypes and their cross combinations for days to calli appearance.

MATERIALS AND METHODS

The anther culture work was carried out in the Molecular Cytogenetics and Tissue culture Laboratory of Department of Crop Improvement, CSK HPKV, Palampur during Rabi 2010-11. The material used and methodology adopted to achieve the objectives of the investigation are given below.The material used for anther culture studies comprised of four elite genotypes and their three cross combinations (Table 1). Sufficient numbers of plants of aforementioned four genotypes and their cross combinations were raised in the pots. In order to have availability of anthers over a long period of time, plants were raised in five lots at an interval of 15 days each. For anther culture, florets from plants were clipped off when the size of bud was about 2-4 mm. The bud size was earlier established on the basis of presence of majority of the microspores at late uninuclate to early binucleate stage as studied by squashing of anthers in a drop of 1% acetocarmine. The florets of appropriate size were collected in 50 ml test tubes containing distilled water.

The florets collected at aforementioned stages were treated with 70% ethanol for 10-15 seconds under aseptic conditions in a laminar air flow chamber. The florets were then surface sterilized with 0.1% $HgCl_2$ for 3-5 minutes with intermittent shaking followed by three washings with sterile distilled water. Florets were blot dried and opened under aseptic conditions with the help of sterile forceps and the six anthers were clipped off from each floret without damaging the anther wall. About 60 anthers were cultured in each pre-sterilized petri plate containing about 25 ml of culture medium. Two basal media *viz.* B_5 (Gamborg *et al.* 1968) and MS (Murashige and Skoog 1962) were used for callus induction. Each of these medium was supplemented with two different sucrose concentrations *i.e.*, 3% and 4% sucrose and each of these sucrose concentrated media was also supplemented with three combinations of hormones *viz.* HM_1, HM_2 and HM_3 (Table 2). All the media were supplemented with 0.8% agar.The experiments on different callus induction media were replicated thrice involving different media and plant growth hormones. Anthers of all four genotypes and their crosses were plated in a replicated fashion. If there was any contamination, replating of the particular treatment was done to complete the experiment under uniform conditions. All the cultured plates were sealed with parafilm wax and kept under dark at 25 ± 1°C until calli were developed. The Days to calli appearance was calculated as follows: Days to calli appearance = Number of days taken for calli appearance

Table 1. List of genotypes and their cross combinations under anther culture study

Sr. No	Genotype	Parentage
1	Jayanti	Developed through irradiation from the parent variety HC-1
2	P-18	Advanced generation mutant obtained through treatment of Jayanti seeds with 0.3% EMS (Pre-Soaked)
3	P-51	Advanced generation mutant obtained through treatment of Jayanti seeds with 0.3% EMS (Pre-Soaked)
4	$P_{(2)2}$	Advanced generation mutant obtained through treatment of Jayanti seeds with 90 kR dose of gamma radiations
5	Jayanti X P-18	-
6	Jayanti X P-51	-
7	Jayanti X $P_{(2)2}$	-

Table 2. Different media, hormones and sucrose concentration used for calli index

Medium	Sucrose Conc.	Hormone	
		Designation	Name and Concentration
B_5	3%	HM_1	NAA (1.0 mg/l)
B_5	3%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
B_5	3%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)
B_5	4%	HM_1	NAA (1.0 mg/l)
B_5	4%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
B_5	4%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)
MS	3%	HM_1	NAA (1.0 mg/l)
MS	3%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
MS	3%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)
MS	4%	HM_1	NAA (1.0 mg/l)
MS	4%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
MS	4%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)

from the day of culturing of anthers. Data on days to calli appearance were analyzed in Factorial Completely Randomized Design (CRD) to obtain the effect of various treatments and their interactions using statistical CPCS software.

RESULTS AND DISCUSSION

Analysis of variance for days to calli appearance involving different parameters is presented in Table 3. Out of four factors, only genotypes had significant effect on

Table 3. ANOVA for days to calli appearance in different genotypes of *Brassica carinata* and their hybrids involving different media, hormones and sucrose concentration

Source of variation	df	Mean Squares	CD (5%)	CV (%)
Genotypes	6	28.23**	0.47	11.00
Hormones	2	1.38	NS	
Genotypes x Hormones	12	5.17**	0.82	
Media	1	0.25	NS	
Genotypes x Media	6	4.67**	0.67	
Hormones x Media	2	21.60**	0.44	
Genotypes x Hormones x Media	12	5.04**	1.16	
Sucrose	1	0.25	NS	
Genotypes x Sucrose	6	0.93	NS	
Hormones x Sucrose	2	63.65**	0.44	
Genotypes x Hormones x Sucrose	12	5.21**	1.16	
Media x Sucrose	1	1.02	NS	
Genotypes x Media x Sucrose	6	12.06**	0.94	
Hormones x Media x Sucrose	2	113.34**	0.62	
Genotypes x Hormones x Media x Sucrose	12	7.19**	1.64	
Error	168	1.02		

**Significant at $P \leq 0.01$

Table 4.1. Effects of media and genotypes on days to calli appearance

Media	Genotypes								
	Jayanti	$P_{(2)2}$	P-51	P-18	Jayanti x $P_{(2)2}$	Jayanti x P-51	Jayanti X P-18	Mean	CD (P≤0.05)
MS	10.50	8.39	9.39	8.44	8.39	9.50	8.44	9.01	NS (Media)
B_5	11.44	11.06	8.56	8.83	8.44	8.89	8.83	9.44	
Mean	10.97	9.72	8.97	8.64	8.42	9.19	8.64		

CD (P≤0.05) = 0.47 (Genotypes)
CD interaction = 0.67 (Genotypes x Media)

Table 4.2. Effects of hormones and genotypes on days to calli appearance

Hormonal combination	Genotypes								
	Jayanti	$P_{(2)2}$	P-51	P-18	Jayanti x $P_{(2)2}$	Jayanti x P-51	Jayanti x P-18	Mean	CD (P≤0.05)
HM_1	13.08	9.75	10.00	10.75	9.33	10.50	10.75	10.60	NS (Hormones)
HM_2	9.67	9.42	7.17	6.67	7.25	7.08	6.67	7.70	
HM_3	10.17	10.00	7.17	8.50	8.67	10.00	8.50	9.00	
Mean	10.97	9.72	8.11	8.64	8.42	9.19	8.64		

CD (P≤0.05) = 0.47 (Genotypes)
CD interaction= 0.82 (Genotypes x Hormones)

Table 4.3. Effects of sucrose and genotypes on days to calli appearance

Sucrose	Genotypes								
	Jayanti	$P_{(2)2}$	P-51	P-18	Jayanti x $P_{(2)2}$	Jayanti x P-51	Jayanti x P-18	Mean	CD (P≤0.05)
3 %	10.17	10.39	8.72	8.56	8.78	9.00	8.56	9.17	NS (Sucrose)
4 %	11.78	9.06	9.22	8.72	8.06	9.39	8.72	9.28	
Mean	10.97	9.72	8.97	8.64	8.42	9.19	8.64		

CD (P≤0.05) = 0.47 (Genotypes)
CD interaction = NS (Genotypes x Sucrose)

days to calli appearance. Nine out of eleven interactions *viz.*, genotypes x hormones, genotypes x media, hormones x media, genotypes x hormones x media, hormones x sucrose, genotypes x hormones x sucrose, genotypes x media x sucrose, hormones x media x sucrose and genotypes x hormones x media x sucrose showed significant effect on days to calli appearance. From the Tables 4.1, 4.2 and 4.3, it is pertinent that the effects of media, hormones and sucrose were found to be non-significant on all seven genotypes which indicated that different genotypes behaved similar in different media, hormonal combinations and sucrose concentrations for days to calli appearance. However, the genotype Jayanti x $P_{(2)2}$ recorded lowest days to calli appearance on different media and sucrose concentrations. Likewise, the genotype P-51 took lowest days to calli appearance on different hormonal combinations. The effects of sucrose and hormones, hormones and media and media and sucrose on days to calli appearance are presented in Tables 5.1, 5.2 and 5.3, respectively. The results revealed that the effects of sucrose and hormones, hormones and

Table 5.1. Effects of sucrose and hormones on days to calli appearance

Sucrose	Hormonal Combination				
	HM_1	HM_2	HM_3	Mean	CD (P≤0.05)
3 %	10.88	7.67	8.95	9.17	NS (Sucrose)
4 %	10.31	7.74	9.79	9.28	
Mean	10.60	7.70	9.37		

CD (P≤0.05) = NS (Hormones)
CD interaction = 0.44 (Hormones x Sucrose)

Table 5.2. Effects of hormones and media on days to calli appearance

Hormonal combination	Callusing Media			
	MS	B_5	Mean	CD (P≤0.05)
HM_1	10.05	10.95	10.50	NS (Hormones)
HM_2	7.40	8.00	7.70	
HM_3	9.38	9.36	9.37	
Mean	8.94	9.44		

CD (P≤0.05) = NS (Media)
CD interaction = 0.44 (Media x Hormones)

Table 5.3. Effect of media and sucrose on days to calli appearance

Media	Sucrose			
	3%	4%	Mean	CD (P≤0.05)
MS	8.98	9.03	9.01	NS (Media)
B_5	9.35	9.52	9.44	
Mean	9.17	9.28		

CD (P≤0.05) = NS (Sucrose)
CD interaction = NS (Sucrose x Media)

media and media and sucrose were found to be non-significant which indicated that days to calli appearance were not affected significantly by different media, hormonal combinations and sucrose concentrations.

CONCLUSION

The factors such as media, hormones, sucrose and their interactions *viz.*, genotypes x sucrose and media x sucrose had non-significant effects on days to calli appearance which indicated that the genotypes behaved similar in different media, hormonal combinations and sucrose concentrations.

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Evaluation of industrial effluent and domestic sewage genotoxicity using *Allium cepa* bioassay

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ABSTRACT

Living organisms including both plants and animals being exposed to polluted water bodies can be impacted. In current investigation the genotoxic effect of industrial effluent and domestic sewage of Barak valley region, Assam, India was investigated using both morphological and root chromosome assay on *Allium cepa*. The mean root lengths of onions exposed to different concentrations of the effluent and sewage (10%, 25% and 50%) were measured for 3 consecutive days for 24, 48 and 72 hrs and the results were compared. The mean root length was statistically evaluated by the analysis of variance. There was both significant increase and decrease in root length among the exposed onion bulbs. Total aberrations increased significantly as concentration increased (p<0.05). Both effluent and sewage samples were recorded to cause harmful damages in the exposed onion test samples. These results demonstrated that the Allium test is a useful screening test for the evaluation of toxicity caused by sewage and effluent samples not only at the morphological level but also at the cytogenetic level; and hence pollution in waster bodies in a major cause of concern. Thus, sincere measures should be undertaken regarding the direct disposable of industrial effluents and domestic sewage and protection of water bodies including its flora and fauna.

KEY WORDS: PAPER MILL EFFLUENTS, DOMESTIC SEWAGE, *ALLIUM CEPA* TEST

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INTRODUCTION

Over two third of Earth's surface is covered by water; less than a third is taken up by land. As Earth's population continues to grow, people are putting ever-increasing pressure on the planet's water resources .In a sense, our oceans, rivers and other inland waters are being squeezed by human activities, not so that they take up less room, but so their quality is reduced. Poorer water quality leads to pollution in water bodies. Water pollution nowadays is a major global problem. It requires ongoing evaluation and revision of water resource policy at all levels. It has been recorded that water pollution is the leading worldwide cause of death and diseases and it accounts for the death of more than 14,000 people daily. India and China are the two countries with high levels of water pollution: An estimated 580 people in India die of water pollution related illness including water borne diseases. Water pollution results in contamination of water bodies like rivers, lakes, aquifer, etc. due to human activities, (Bennet, 1997, Prasad and Rao, 2010. Bakare et al, 2017, Anacleto et al, 2017).

Polllution in water bodies occurs when contaminants gets introduced into the natural environment. For example, releasing inadequately treated waste water into natural water bodies leading to degradation of aquatic ecosystem. This in turn can cause public health problems for people living in downstream as people are using this polluted water for regular domestic activities like bathing, washing, drinking, irrigation, etc. Researches have revealed that the sources of water pollution may be point sources and non-point sources. Point sources have an identifiable cause as storm drain, waste water treatment plant or streams. Non-point sources are more diffuse like agricultural runoff, (Zaiad, 2010). With the increasing development of industrial resources the risk of water pollution has also increased. Not only the industrial resources but also the inadequate system of dumping of municipal sewage has resulted in pollution of water bodies and its aquatic ecosystem including both aquatic flora and fauna.

Researches till date have revealed that dissolved contaminants in both effluents and sewages when exposed to water bodies not only harms plant growth but also forces plants to absorb dangerous chemicals and pollutants which gets passed to animals and human through consumption and other modes (Sik *et al,* 2009). In a work done on the variable actions of *Alium sepa* and its usage as a bio indicator of cadmium toxicity, plants were exposed to increasing concentrations of cadmium, where cadmium was observed to cause inhibition of root and leaves growth and elongation which serve as a tool for characterizing the bio indication of cadmium exposure in waste and effluent condition, (Bakare et al, 2017).

The direct application of industrial sludges were recorded to harm the local biota in an investigation where the genotoxicity of industrial sludges was assessed using various plant including *A.cepa* where *A.cepa* test was found to be effective in detection of damages (Anacleto et al, 2017).

Studies about phytotoxic effects of waste waters and effluents started in 1970s where researches were conducted taking sugar cane, eucalyptus, *Triticum aestivum*, *Brassica campastris*, Sorghum, rice, and many more. *Allium cepa* is the largest genus of petaloid monocotyledons, containing hundreds of species naturally distributed in temperate climates of the northern hemisphere (Koçyi ğit & Özhatay, 2010). This test has important advantages (Zegura, 2009) and has been used from many years in investigating physical and chemical mutagenesis and cytogenetic effects in mitotic cell division. *Allium cepa* is important since it is an excellent model in-vivo, where the roots grow in direct contact with the substance of interest enabling possible damage to the DNA of eukaryotes to be predicted. It is advantageous to use the *Allium cepa* test system since its main component is a vascular plant, making it an evaluating environmental pollutant , detecting mutagens (Gupta et al, 2009).

The present investigation was designed to examine the level of morphological and genotoxic damages caused by industrial effluents as well as domestic sewages on *Allium cepa* so that proper safety measures can be taken not only for the protection of water quality but also preventive measures can be taken against the damages caused to aquatic ecosystem prior to exposure of effluents and sewage.

MATERIALS AND METHODS

For present investigation raw paper mill effluent sample was collected from the outlet pipes in the local river Barak of the valley. The domestic sewage which was selected for comparative analysis was collected from Silchar, Municipal drainage system at Tarapur area, where all the debris and discharges of the whole locality have been found to be discharged. Both effluent and sewage samples were collected in plastic gallons, pH was measured and stored at -20°C to prevent further microbial growth.

The common purple onion, *Allium cepa* (2n=16) bulbs (1.5–2.0 cm diameter) used for this study was procured from organic farmers of almost equal weight age. The dried out scales were carefully removed leaving the ring of the root primordial intact (Fiskesjo, 2011). Then they were kept in moist condition to let root grow for three days, this help select onion with synchronous growth. For each test, 10 *A. cepa* bulbs purchased from organic farmers were set up to produce roots in filtered and dechlorinized tap water for three days and then

transferred to the test solutions. Tap water was previously filtered in a bio-activated coal filter to remove chlorine and its by-products commonly used for disinfecting drinking water. Three litres of water were aerated over a period of 24 h before filling the test tubes. For positive control mitomycin C was selected . Mitomycins are a family of azinidines containing natural products insulated from *Streptomyces lavendulae*. Mitomycin C is a potent DNA crosslinkers. A single cross link per genome has shown to be effective in killing bacteria. This is accomplished by reductive activities followed by 2 N –Alkylation. Both alkylation are sequence specific for a guanine nucleotide sequence.

Three different concentrations of both the effluents and sewage were selected as 10%, 25% and 50% for exposure through prior standardization. During the Allium cepa assay, all selected onions were exposed to the selected concentrations of effluent and sewage for 24hrs, 48hrs and 72 hrs, respectively. The growth in roots were recorded till the third day of exposure in water and after that the variation in root growth were recorded after every 24 hrs for next three consecutive days till 72hrs and the data were recorded and compared. For mitotic studies, growth inhibition tests were carried out for each sample, to find its toxicity level. After every 24hrs of exposure, 3 to 4 healthy root tips from each bulb were prepared for the microscopic slides.

The emerged root tips of the onion bulbs in the different concentration of sewage and effluent were fixed and macerated in a solution of 45% acetic acid (9 parts) and 1 N HCl (1 part) at 50 °C for 10 min, followed by squashing in 2% Acetocarmine stain for 15 minutes. The modified conventional Feulgen-squash method (Sharma and Dphil, 2012) was used to prepare permanent slides of root meristems. The root tips were put in 1 normal hydrochloric acid for five minutes to soften the tissue. The macerated and stained root tips were covered with cover slip and squashed. Minimum 3-4 Slides were prepared per bulb for microscopic observation. Approximately three thousand cells were examined per onion to remove the errors and classified according to the chromosomal aberrations presented including bridges, fragments and chromosome lagging.

Results were presented as Mean±SE where mean value was calculated from three individual readings of a particular set. ANOVA was performed to determine the level of significance from the set of onion bulbs. ANOVA was done using graph pad PRISM (Graph pad Inc., san Diego, CA, USA).

RESULTS AND DISCUSSION

Water pollution can be caused by a number of sources ranging from industrial resources and sewage treatment plants and factories to mining activities, paved roads and agricultural runoff. Such issues have become one of the biggest problems in many developing and developed countries. These pollutants when not treated properly, can cause mutagenic or toxic effects directly on humans, affecting human health, resulting in diseases like cancer, congenital malformations, and cardiovascular diseases (Grover & Kauer, 2009). Siddiqui and his group (Siddiqui et al., 2011) have worked to validate plant-based tests for assessing the toxicity of water in India.

The *Allium* test is advantageous as genotoxicity screening assay, as *Allium* root cells posses the mixed function oxidase system which is capable of activating promutagens or genotoxic chemicals (Odeigah *et al*; 1997a). In the *Allium* test, inhibition of rooting and the appearance of stunted roots indicate retardation of growth and genotoxicty, while root wilting explains toxicity (Odeigah *et al*; 1997b). Both growth retardation and root wilting are accompanied by suppression of mitotic activity and remarkable chromosomal aberration. The present findings provides evidence that effluent and sewage inhibited root growth and caused growth retardation. The reason behind growth inhibition may be due to high rate of chemical oxygen demand which affected certain physiological processes leading to the disturbance in the balance between promoter and inhibitors of endogenous growth regulator (Gill and Saggoo, 2010).

Growth inhibition was most recorded at 50% concentration along with a marked decrease in root length when compared with the control. This is usually accompanied by an increase in chromosome aberrations (Amin and Muzahid, 2009). The suppression of mitotic activity was often used in tracing cytotoxicity (Smaka-Kinel *et al.,* 2013). In our study a decrease in the mitotic index was found as the concentration of effluent increased which indicates the cytotoxic effect of sewage and industrial effluent. Chrosomal aberration were observed to increase as the concentration of effluent and sewage increased. Among the chromosomal aberration observed, IN, EN and CF were most frequent in all concentrations band kept on increasing from concentrations of industrial effluent towards higher concentrations of sewage. Such findings are responsible for the completely decayed roots found in 25% and 50% concentration. The most common abnormalities were c-mitosis and disturbed metaphase. Sticky chromosomes and binucleated cells were recorded in noticeable amount. In addition to the above, at anaphase and telophase bridges, lagging chromosomes and irregular anaphase were also observed. The mitotic index in the root meristems grown in the negative control ranged from 17.3 to 19.8.

Table 2 shows the mitotic index values in root meristems growth in different concentrations of effluent

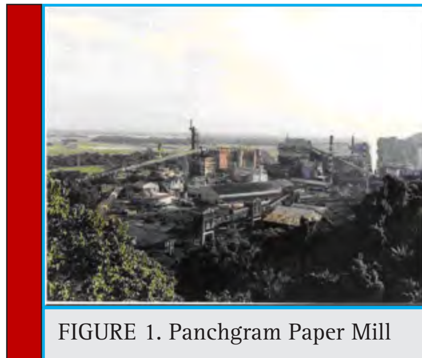

FIGURE 1. Panchgram Paper Mill

FIGURE 2. Silchar Municipal sewage

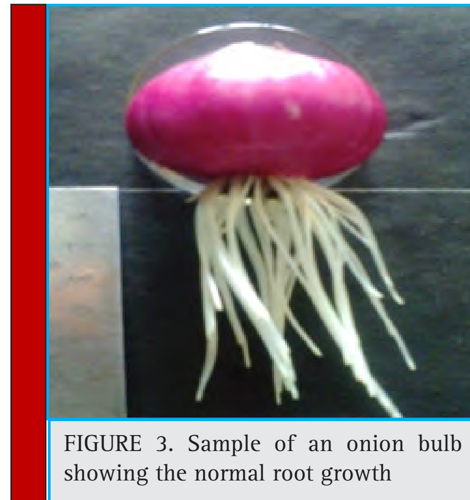

FIGURE 3. Sample of an onion bulb showing the normal root growth

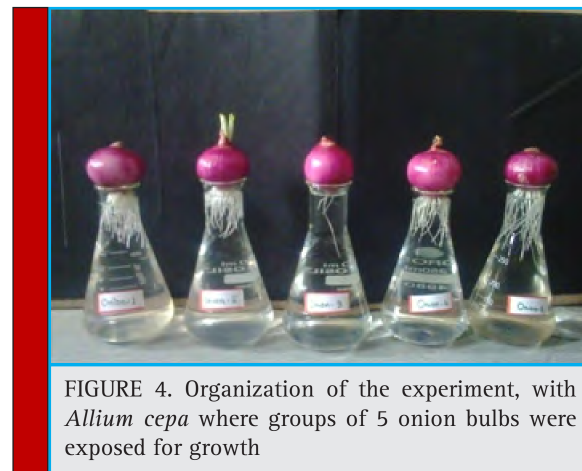

FIGURE 4. Organization of the experiment, with *Allium cepa* where groups of 5 onion bulbs were exposed for growth

and sewage from the three concentrations of wastewater treatment. The decrease in the mitotic index of the root tips reached statistical significance only in the highest tested concentrations. The cytogenetic aberrations most commonly observed in anaphase-telophase cells were bridges, fragments and chromosome lagging. Aberrant mitotic cells were counted and expressed as mean and SD of the selected onion bulbs. In the root meristems of the negative control, the percentage of aberrant cells

Table 1. Root length variation of *Allium cepa* after cultivation in different concentrations of paper mill effluent and domestic sewage (10%,25% and 50%)

Treatment gropus	**Concentration**	**Root length in different time interval (mean±std.error)**					
		Before treatment			**After treatment**		
		24 hrs	**48 hrs**	**72hrs**	**24 hrs**	**48hrs**	**72 hrs**
Control	---	0.16±0.045	0.8±0.078	3.67±0.136	6.04±1.34	7.57±1.44	9.04±1.65
Positive Control (MMC)	2mg/lit	1.64±0.22	2.97±0.37	4.57±0.93	5.54±0.59	5.7 ±0.55	5.77±0.62
Paper Mill Effluent	10%	0.26±0.075	1.1±0.129	4.44±0.062	$5.03^{***}\pm0.045$	$5.13^{***}\pm0.045$	$6.1^{**}\pm0.107$
	25%	0.2±0.068	1.23±0.091	4.06±0.39	$5.05^{***}\pm0.35$	$4.86^{***}\pm0.349$	5.36±0.286
	50%	0.36±0.045	1.67±0.169	2.96±0.223	$3.9^{***}\pm0.223$	$4.53^{***}\pm0.075$	4.93±0.062
Domestic Sewage	10%	0.23±0.062	1.93±0.219	4.13±0.164	$4.8^{***}\pm0.165$	$5.3^{***}\pm0.165$	5.7±0.186
	25%	0.13±0.068	1.23±0.091	4.33±0.169	$4.9^{***}\pm0.181$	$5.3^{***}\pm0.181$	5.66±0.198
	50%	0.35±0.062	1.73±0.248	3.73±0.091	$3.96^{***}\pm0.075$	4.3(((±0.029	4.5±0.029

Root length unit=cm; n=3.

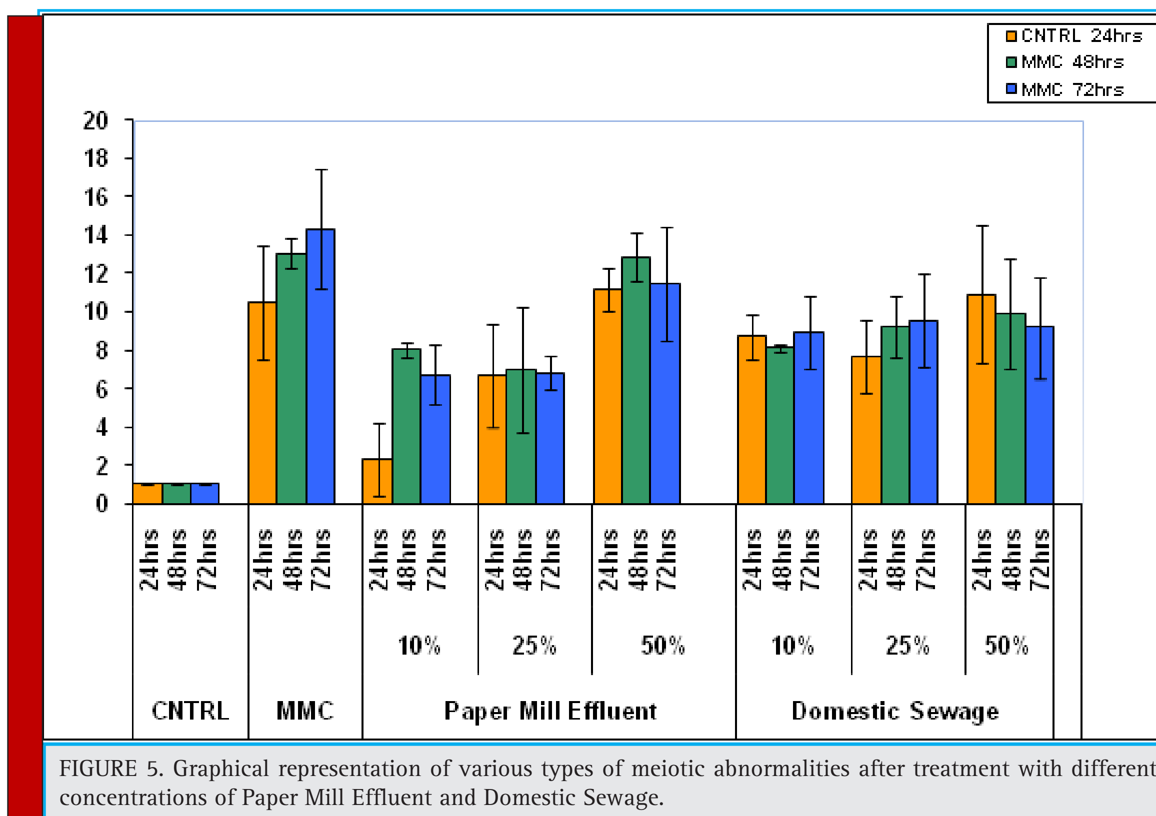

FIGURE 5. Graphical representation of various types of meiotic abnormalities after treatment with different concentrations of Paper Mill Effluent and Domestic Sewage.

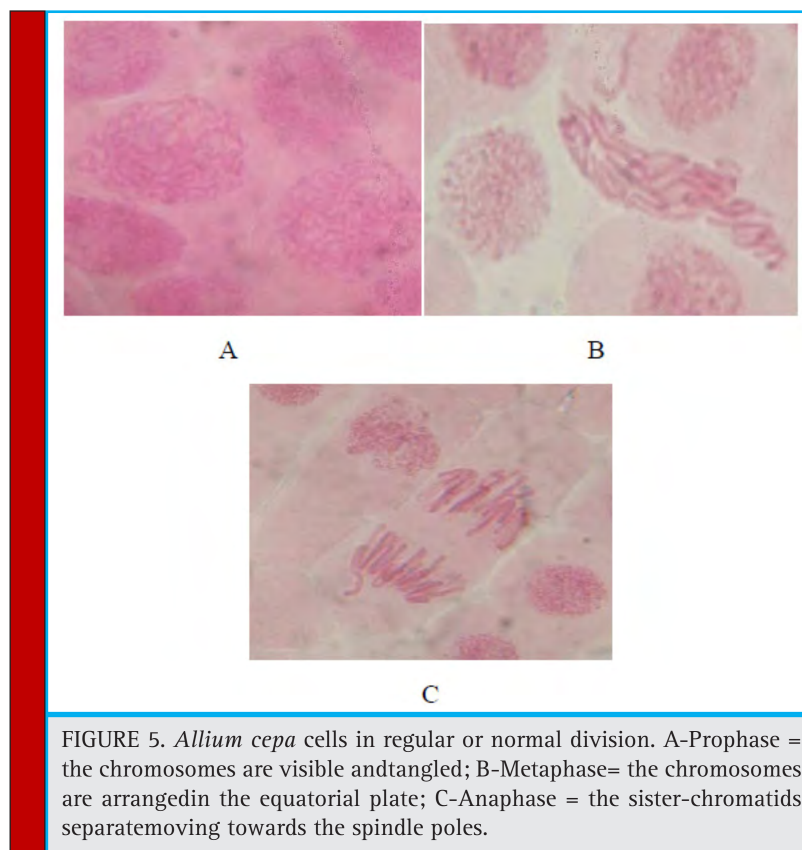

FIGURE 5. *Allium cepa* cells in regular or normal division. A-Prophase = the chromosomes are visible andtangled; B-Metaphase= the chromosomes are arrangedin the equatorial plate; C-Anaphase = the sister-chromatids separatemoving towards the spindle poles.

Table 2. Frequencies of different types of meiotic abnormalities after treatment with different concentrations of Paper Mill Effluent and Domestic Sewage.

Dose/Treatment		Fixn. time	Total no. of cells (n=3)	No. of dividing cells	MI (mean±std. deviation) MN	Cytotoxic effects								Total Aberrant Cells	% Aberrant Cells (mean± std.error)
						STK	IN	EN	VC	BNC	CB	CF			
Contrlo		24h	3060	542	17.34±2.10	0	0	0	0	0	0	0	0	0	1.02±0.02
48h		3060	610	19.66±1.96	0	0	0	0	0	0	0	0	0	1.03±0.05	
72h		3060	623	19.8±1.56	0	0	0	0	0	0	0	1	1	1.03±0.05	
Positive control (MMC)		24h	3080	248	8.04±0.44	15	22	85	15	3	2	5	22	323	10.48±2.98
48h		3099	224	6.8±0.30	26	24	98	18	6	6	16	21	404	13.03±0.78	
72h		3119	246	7.87±0.28	33	28	101	22	8	8	23	18	447	14.33±5.08	
Paper Mill Effluent	10%	24h	3061	491	16.36±0.14	0	30	93***	15	12	13	17	51	231	2.31**±1.86
		48h	3083	450	14.57±3.48	0	19	133***	6	4	3	11*	71***	247	8.01***±2.42
		72h	3075	472	15.33±2.19	0	16	156***	9	10	4	9	69***	273	6.73**±2.59
	25%	24h	3061	429	14.03±1.08	0	16	80***	20	15	10	17	46	204	6.72**±3.68
		48h	3050	461	15.11±0.11	0	20	122***	18	18	20	13	22	233	6.98**±3.24
		72h	3020	456	15.09±0.22	0	17	85***	20	20	21	22	21*	206	6.82**±0.86
	50%	24h	3072	337	10.97±0.9	0	56***	127***	27	22	17	14	80	343	11.16***±4.13
		48h	3067	408	13.29±0.84	3	59***	156***	39	25*	24	20	82***	408	12.87***±2.27
		72h	3033	437	14.42±1.4	3**	80***	145***	40	37***	19	30	98***	452	11.5***±6.98
Domestic Sewage	10%	24h	3031	359	11.85±1.1	0	0	94***	137***	1	24	5	3	264	8.7**±1.2
		48h	3051	436	14.29±1.49	0	0	92***	110***	1	36	6	3	248	8.12**±0.17
		72h	3063	512	16.34±1.22	0	1	96***	116***	3	36	13	10	275	8.94**±1.89
	25%	24h	3044	551	18.07±3.66	0	15	66*	66**	8	24	26*	34	239	7.65**±1.91
		48h	3089	617	27.56±10.49	0	25	60*	66**	26	34	42***	33	286	9.22***±1.64
		72h	3051	652	21.38±1.28	0	30	67**	72**	32***	30	24	34	289	9.55***±3.44
	50%	24h	3023	431	14.25±0.58	2	13	72***	115***	22	44	33***	30	331	10.91***±5.61
		48h	3046	457	14.66±1.46	1	35*	55*	6**8	26*	31	42***	43	301	9.88***±4.88
		72h	3045	473	15.58±1.57	4	57***	34	16	36***	20	57***	55*	279	9.19***±3.63

MN=Micronucleus, STK=Stickiness, IN=Irregular Nuclues, VC=Vagrant Chromosome, BNC=Bi Nucleated Cells, CB=Chromosome Bridge, CF=Chromosome Fragment.
Control : Dechlorinated tap water, MMC: Mitomycin C was used as positive control, When compared PME and DS with Control $P<0.05$=* $P<0.01$=**; $P<0.001$=***

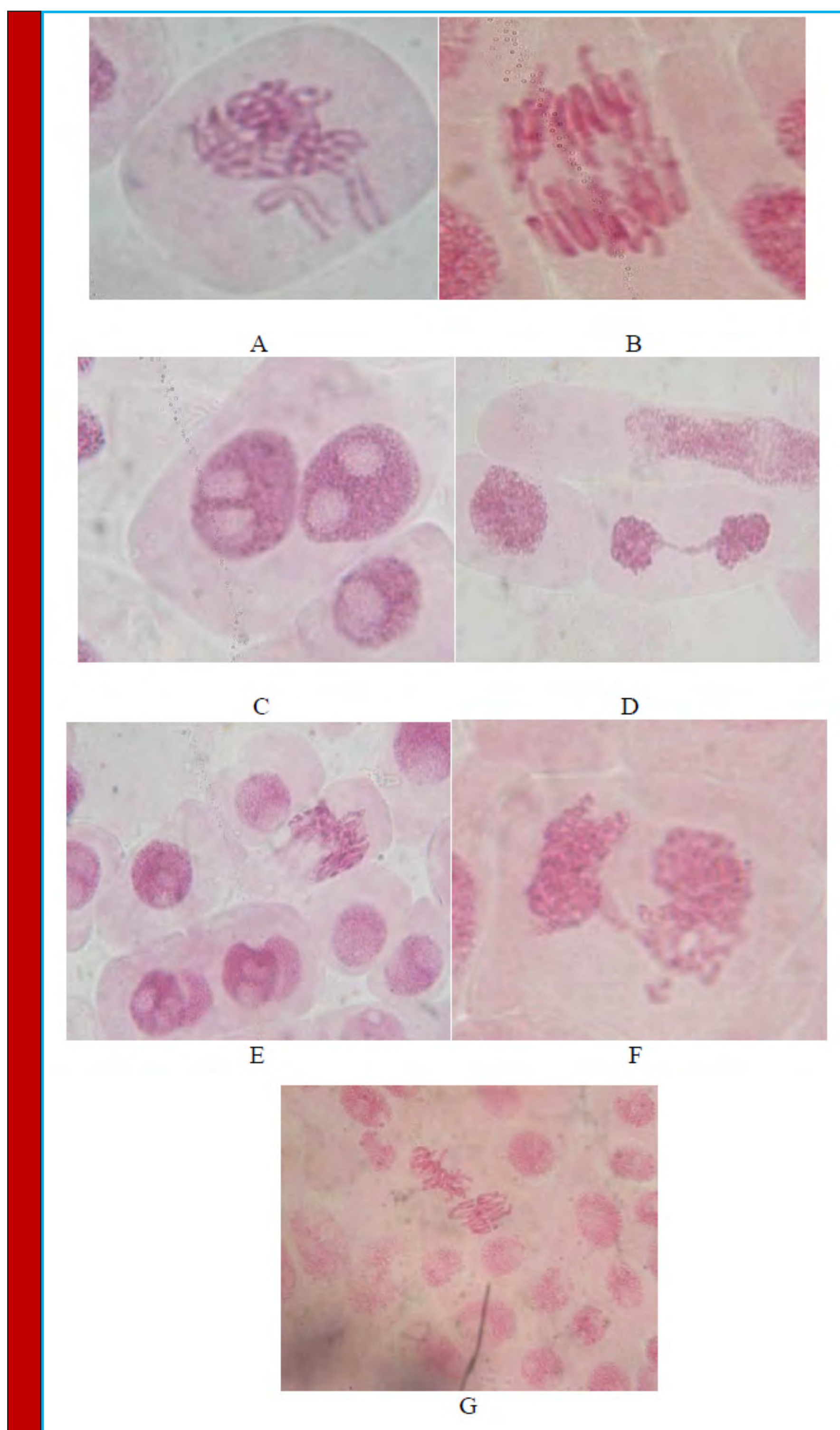

FIGURE 6. *Allium cepa* meristematic cells showing the alterations due to the action of industrial effluent and domestic sewage; A-irregular metaphase, with unorganized chromosome, alsoknown as C-metaphase, showing chromosomes with no orientation on the equatorial plate; B-irregular anaphase,with anaphasicmicro-bridges; C-irregular cell, binucleate, with an elliptical aspect; D-telophase bridge; E-cell with adherent or damaged nucleus, F-irregular cell; G-metaphase with numerical alteration, due to duplication of the number of Chromosomes.

H I

FIGURE 7. Photographs of onion bilbs captured after exposure to 50% concentration of effluent and sewage

represents an avg. of 1.03 which was recorded as 8.34 and 10.09 in effluent and sewages.

Results obtained in our study showed that sewage and effluent induced chromosomal aberration through interactions with DNA and proteins leading to chromosome stickiness, mitotic disturbances and cell damage. Present finding is similar to the results of Odeigah *et al.* (1997b) in which roots of *Allium cepa* exposed to effluent from industrial wastes resulted to cells with chromosomal aberration. It would be beneficial to apply *A. cepa* chromosomal assay as a tool for monitoring the genotoxic effects of industrial effluent and wastewaters thereby providing information on the need for environmental managers to further subject treated industrial effluent to Toxicity Identification Evaluation and Toxicity Reduction Evaluation before they are finally discharged. This will enable proper chemical analysis of industrial effluent in order to identify the constituent that is really genotoxic and its prompt removal from the effluent before discharge.

CONCLUSION

According to the environmental campaign organization WWF," Pollution from toxic chemicals threatens life on this planet. Every ocean and every continent, from the tropics to the once-pristine polar regions, is contaminated. Clearly the problems associated with water pollution can disrupt life on our planet to a great extent. Increasing population and industrialization and direct exposure of sewage in water bodies has resulted in production of huge amount of liquid waste which ultimately finds its way to water bodies like rivers, lakes, streams and oceans. These effluents and sewages if provided to plants, animals and human for consumption, can lead to spreading of different types of hazardous diseases. Hence, it becomes mandatory to analyze the level of pollution caused to water and its flora and fauna.

We can conclude from our investigation that *Allium cepa* test is an excellent biomarker of chromosomal aberrations and has occupied an important place for the prevention and prediction of environmental impact that will be caused by the exposure of effluents and sewages. The above findings revealed the fact that industrial effluent and domestic sewage have almost equivalent capabilities to cause harmful damages to the water bodies. Life is ultimately about choices and so is pollution. We can live with sewage strewn beaches, dead rivers, and consume fish that are too poisonous for us to eat. Or we can work together to keep the environment clean so that plants, animals and people who depend on it remains healthy. It is ultimately up to us to be informed, responsible and involved when it comes to the problem we face with our water. We must become familiar with our water resources and learn about ways for disposing harmful household wastes so that they do not end up in sewage treatment plants that can't handle them or landfills not designed to receive hazardous materials. Hence proper measures should to be taken for the management of water quality and the aquatic life.

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Optimization of sampling size for DNA-based PCR assay for hybrid purity test in the brinjal, *Solanum melongena*

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ABSTRACT

Farmers can harness the full potential of any hybrid only when they get genetically pure seeds of the hybrid. Hence, ensuring the genetic purity of certified seeds of brinjal hybrids is mandatory in India, which is done through field grow out test (GOT) based on the morphological characters of plants grown to maturity. GOT being land and labour intensive, time consuming and influenced by the environment, there is a need to identify rapid and reliable alternatives like DNA based assays. Therefore, the present study was undertaken to identify the SSR markers that could be used to test the hybrid purity of two commercial brinjal hybrids (viz., Arka Anand and Brinjal Asha) and to optimize the minimum sample size that can be used for purity assessment of the brinjal hybrids. Among 120 SSR markers studied, two markers were found to be suitable for testing the purity of these hybrids. The analysis of plant-to-plant variation within the parental lines of all the hybrids, using the identified hybrid specific markers, showed highly homogenous SSR profile, which further indicated the scope of application of these markers in maintenance and purity testing of hybrids and parental lines. These two co-dominant markers can be used as referral markers for unambiguous identification, seed purity testing and protection of the hybrids.

KEY WORDS: BRINJAL, CO-DOMINANT, GOT (GROW OUT TEST), HYBRID PURITY, SSR

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INTRODUCTION

Brinjal (*Solanum melongena* L.) identified as eudicot, warm weather crop majorly grows in tropical and sub-tropical regions of the world and is also known by name eggplant (Schippers 2000; Daunay and Hazra, 2012). Brinjal remains fifth most valued crop of *Solanaceous* with commercial value of US $10 billion a year after potato, tomato, pepper and tobacco (FAO, 2014). India is the major Brinjal producer with annual production reaching 13.55 million tonnes with a 19.1 mt/ha productivity (NHB database, 2014). Brinjal also remains rich in nutritional contents such as vitamins, minerals and bioactive materials which supply number of benefits to human beings, (Raigon et al., 2008; Plazas et al., 2014b; Docimo et al., 2016).

Although eggplant remains the promising crop for productivity it remains susceptible to number of plant pathogens such as bacterial wilt, fusarium wilt and many others (Rotino et al., 1997). It is also being used as nutrient source by number of pests such as shoot borer, white-flies, mites, aphids and others (Medakker and Vijayaraghavan, 2007; Rotino et al., 1997). In an attempt related to crop improvement, number of breeding programmes are involving new characters to create promising varieties as F1 generation imparting better shelf life, disease resistance and nutritional qualities (Daunay and Hazra, 2012). In many seed industries, Hybrid seed production of the Brinjal is carried out by hand emasculation and pollination; still brinjal undergoes cross-pollination to change the genetic purity by involving foreign pollens.

The traditional Grow Out Test (GOT) is based on the genetically induced morphological and biochemical features expressed at suitable stages of development. Grow out test (GOT) is an important method for genotypic identification through adult plant phenotype (Arus, 1983). The physical features are studied in detail and all the other plants i.e. off-types, pollen shredder, objectionable and diseased plants, etc are traced out. GOT has got certain limitations, as it is an expensive procedure. GOTs take up a full growing season, which often results in late entry of seed in market. In addition to GOT, number of biochemical markers are used such as isozymes and seed storage proteins, but they also remained restricted in their usage (Dadlani et al., 1997; Mehetre and Dahat, 2001; Borle et al., 2007 and Rakshit et al., 2008). Keeping in view, scientific community is now moving towards DNA markers which provides diversity in approach with the type of methodology adopted such as simple sequence repeats (Rana, 2003; Dongre and Parkhi, 2005; Saravanan et al., 2007), Amplified fragment length polymorphism (Rana and Bhat, 2004), Random amplified polymorphic DNA (Geng et al., 1995; Venu, 2001, Rao et al., 2002). Restriction fragment length polymorphism (Pendse et al., 2001; Dongre and Parkhi, 2005) and ISSR marker (Dongre and Parkhi, 2005; Rana, 2006). These are now widely used in hybrid purity check program.

Among all DNA markers, now SSR markers are found to be promising with its co-dominant, polymorphic, discriminative, reliable and repeatable features and can be standardized for Distinctness, Uniformity and Stability (DUS) testing (UPOV, 1997). This marker (SSR) undoubtedly overcome the problem of errors in morphological based selections of hybrids which is occurring due to plant growth stage and environmental fluctuation.Even though molecular marker plays such an important role in identifying the hybrid purity, there are no guidelines available on the use of specific number of markers & sample size for 90, 95, & 99% purity in comparison with the GOT (Grow-Out Test), where 400 seeds were used for 95% purity. There is an urgent need for this method to be standardized for large scale "Hybrid Purity Test". Hence, the present study was undertaken with the objective of optimizing the minimum sample size that can be used for purity assessment of the brinjal hybrids. The results obtained using SSR markers were then compared with those from a GOT performed on the various sample size and the percentage of genetic purity was calculated for both GOT and SSR analysis.

MATERIALS AND METHODS

PLANT MATERIALS

The present study was carried out at Indian Institute of Horticultural Research, Bangalore, India during 2014-2016. Two commercial F_1 hybrid brinjal cultivars were tested. Cultivar 'Arka Anand' and its parental (female and male) lines were developed in Indian Institute of Horticultural Research, Bangalore, India while 'Brinjal Asha' and its related parental (female and male) lines were provided by Noble Seeds Pvt. Ltd., Bangalore, India.

GROW OUT TEST (GOT)

To validate the conformity of the molecular marker-based estimates of selfed or off-type or outcrossed plants with the actual field morphological data, the experiment was conducted by pooling 95% F_1 hybrids mixed with 5% off-types / admixture individually in the following sets of 400, 300, 200 and 100. The parental and four set of seed lots from each hybrid were sown in the greenhouse with a day temperature of 24 $\pm$ 3 ^{o}C and a night temperature of 18 $\pm$ 3 ^{o}C in the Indian Institute of Horticultural Research, Bangalore. One-month-old seedlings were transplanted to the polyhouse in 5m row with 10cm plant to plant and 45cm row to row spacing. Regular irrigation, fertilization, staking and crop protec-

tion measures were adopted, and purity visual evaluation was conducted based on the important morphological characters throughout the growth period. The details on morphological traits that have been recorded to distinguish the true hybrids from off types for all the two hybrids along with their parents are furnished in table 1. The genetic purity of hybrids was calculated as:

$$\text{Hybrid purity (\%)} = \frac{\text{((Total number of plants-number of off types))}}{\text{(Total number of plants)}} \times 100$$

DNA EXTRACTION

Total genomic DNA was extracted from young and fresh leaves using modified CTAB method (Doyle & Doyle, 1990). The quality and quantity of isolated genomic DNA was checked using 0.8% Agarose gel electrophoresis and Gene Quant UV Spectrophotometer (GE Health Care Bio-Sciences Ltd., Bengaluru, India) respectively. After quantification, the final concentration of DNA was adjusted to 20ng/µl and used as template DNA for PCR amplification.

PCR AMPLIFICATION PROFILE

A total of 120 SSR markers were screened for the detection of polymorphism within the parents of Arka Anand and Brinjal Asha. Later the polymorphic SSR markers were tested on F_1 population to determine the discriminatory and stability of F_1 plants. The PCR assay was performed in a 25µl volume containing 2.5µl of 10X PCR buffer, 2.5 µL of 20mM $MgCl_2$, 2.5µL of 1mM dNTP mix, 2.5µL of 5pmol of each (F&R) primer, 2.5µl of 20ng/µl of template DNA, 0.3µl of 3 units of Taq DNA polymerase and 9.67µL of double distilled water to a total volume of 25µL. The amplification was carried out in a thermocycler (model TC-5000; Bibby Scientific (Asia Limited, Hong Kong). A touch down PCR protocol was optimised in brinjal with a temperature program consisting of the initial denaturation at 95 °C for 30 minutes followed by 10 cycles with a decrease of 1 °C per cycle of denaturation at 94 °C for 30 seconds, annealing at 60-55 °C for 30 seconds and polymerization at 72 °C for 1 minute followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing

Table 1. Morphological characters used to identify the selfed/offtypes during the Grow- out Test.

Brinjal (Arka Anand)				
Sl. No.	**Morphological characters**	**Female parent**	**Male parent**	**Hybrid (Brinjal Asha)**
1.	Plant: Growth habit	Semi erect	Semi erect	Erect
2.	Stem hairiness	Present	Slightly present	Present
3.	Leaf hairiness	Present	Absent	Slightly Present
4.	Leaf colour	Dark green	Light green	Light green
5.	Flower:colour	Dark purple	Dark purple	Light purple
6.	Flower:petal no.	5	6	6
7.	Calyx:spininess	Present	Absent	Absent
8.	Fruit:colour of calyx	Purplish	Greenish	Light Greenish
9.	Fruit: General shape	Obovate	Ovoid	Ovoid
10.	Fruit: Colour	Dark purple	Dark purple	Light purple
11.	Fruit: Stripes	Absent	Present	present
Brinjal (Asha)				
Sl. No.	**Morphological characters**	**Female parent**	**Male parent**	**Hybrid**
1.	Plant: Growth habit	Semi erect	Semi erect	Erect
2.	Stem hairiness	Present	Slightly present	Present
3.	Leaf hairiness	Present	Absent	Slightly Present
4.	Leaf colour	Dark green	Light green	Light green
5.	Flower:colour	Dark purple	Dark purple	Light purple
6.	Flower:petal no.	5	6	6
7.	Calyx:spininess	Present	Absent	Absent
8.	Fruit:colour of calyx	Purplish	Greenish	Light Greenish
9.	Fruit: General shape	Obovate	Ovoid	Ovoid
10.	Fruit: Colour	Dark purple	Dark purple	Light purple
11.	Fruit: Stripes	Absent	Present	present

at 55 °C for 30 seconds and polymerization at 72 °C for 1 minute. Final elongation was at 72 °C for 5 min.

The amplification products were analyzed by electrophoresis in a 3% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide in 45 mM Tris-borate- (1mM) EDTA buffer, pH 8.0. 25 µl of PCR products was loaded into the well after adding 3µl of loading dye (50% (w/v) glycerol and 50% (w/v) BPB). The 100bp DNA ladder (3B bioscience, Spain) was used to calculate PCR product size. Electrophoresis was carried out at 70V for 1hr. The amplified fragments were visualized with UV transilluminator (Syngene, USA) and documented using UV-Pro gel documentation system.

RESULTS AND DISCUSSION

In the Grow-out Test, purity evaluation was conducted based on morphological traits. In the present study, plants from four different sample sizes (400, 300, 200 and 100) of 'Arka Anand and Brinjal Asha' were studied individually to determine if they were true-to-type for ten morphological characters in the GOT (Table 1). In case of Arka Anand, out of the ten morphological characters analysed; Flower petal no., leaf colour, leaf length, fruit shape and fruit colour exhibited the maximum variation (Figure 1). The percentage of hybrid purity in GOT assay for 400, 300, 200 and 100 sample sizes of Arka Anand was calculated to be 95% (Table 2). In case of Brinjal Asha, out of the ten morphological characters analysed; flower colour, flower petal no., calyx spininess, fruit shape, colour of calyx, fruit colour and presence of fruit stripes exhibited the maximum variation (Figure 2). The characters of few individuals shown deviation from the standard characters were identified as off-type and they were similar to those of the female parental type. The percentage of hybrid purity in GOT assay for 400, 300, 200 and 100 sample sizes of Brinjal Asha was calculated to be 93%, 95%, 95%, 94% respectively (Table 2).

In the PCR-based assay, a total of 120 pairs of SSR markers were tested, with twenty-one markers

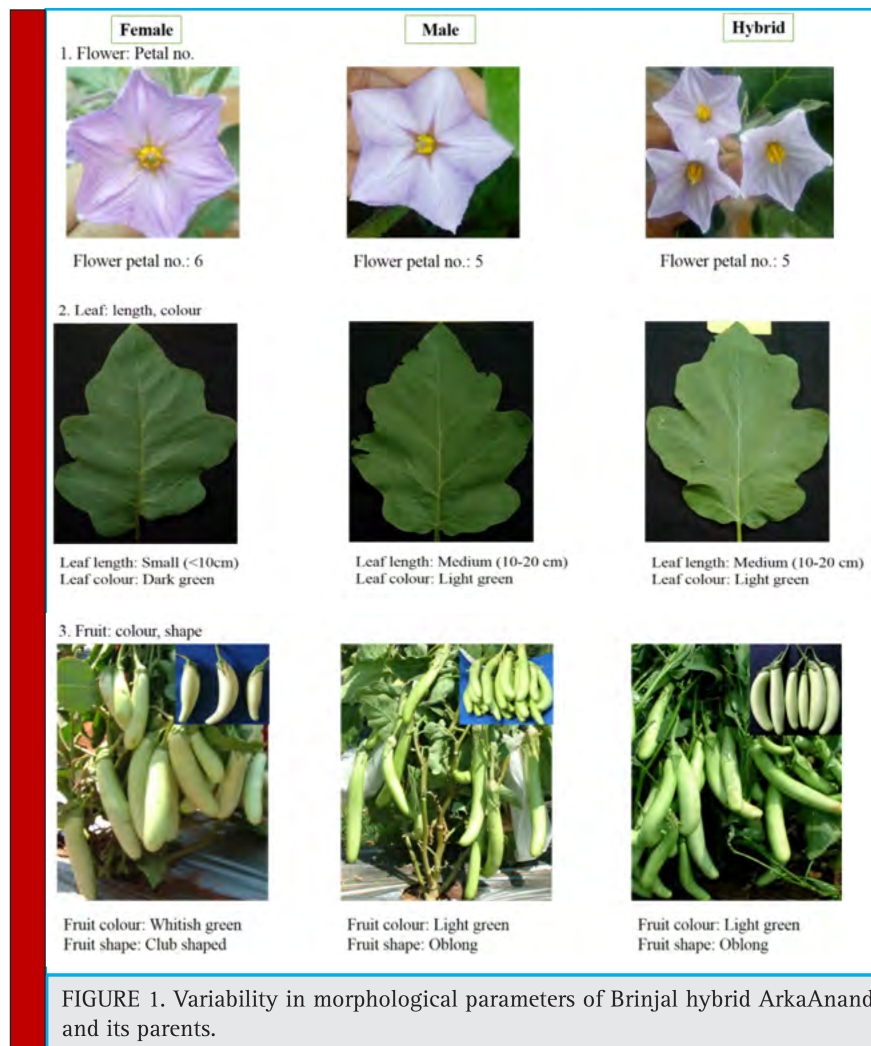

FIGURE 1. Variability in morphological parameters of Brinjal hybrid ArkaAnand and its parents.

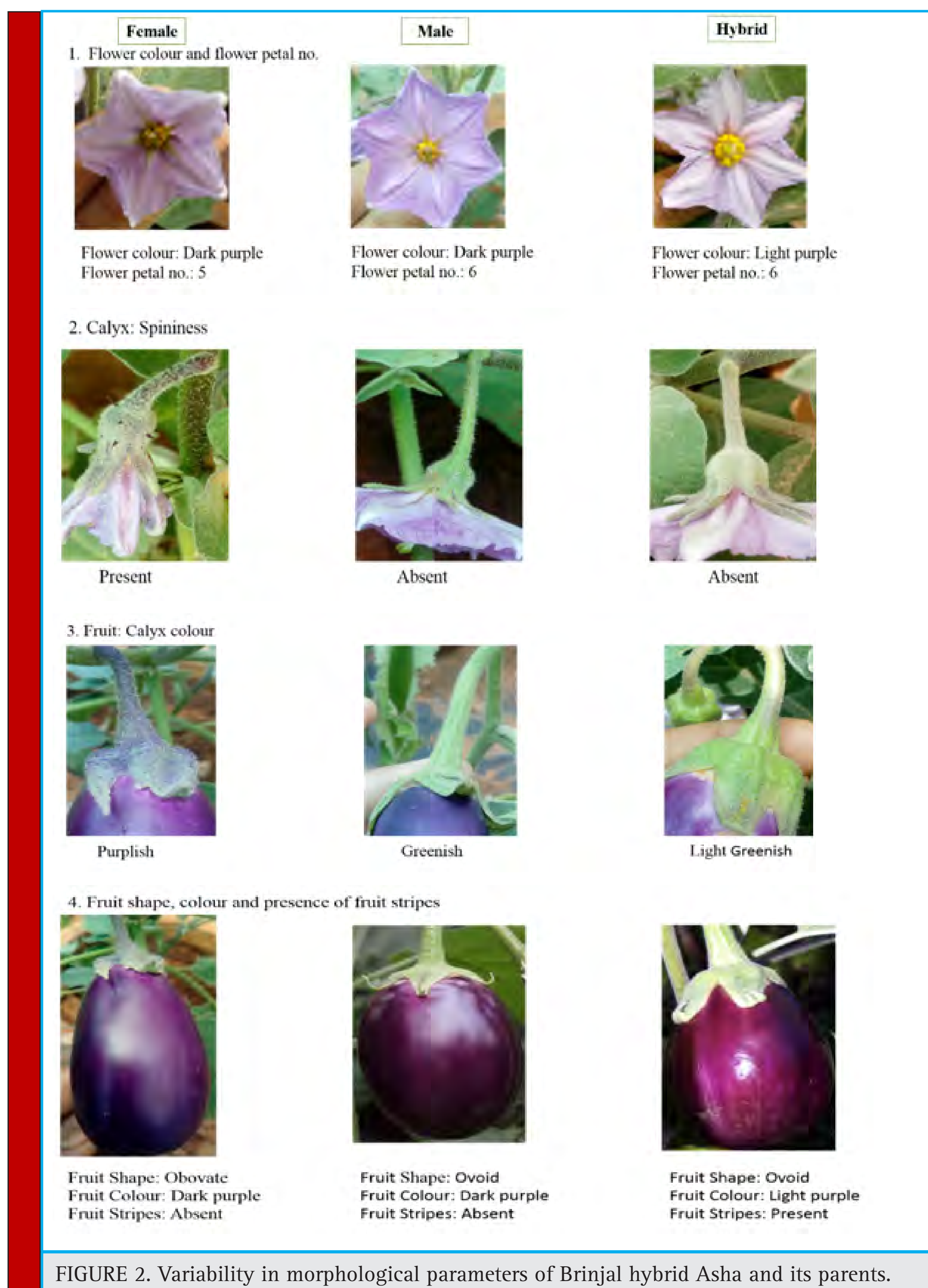

FIGURE 2. Variability in morphological parameters of Brinjal hybrid Asha and its parents.

(Table 3) showing polymorphism between the parental lines. Out of 21 polymorphic markers, two SSR markers (**eme08D09** & **emb01F16**) was found to be Co-dominant. Using **eme08D09**, we were able to determine hybrid purity of two Brinjal hybrids (Arka Anand & Brinjal Asha) resulted in amplification of 210bp female-specific amplicon (FSA) as well as 230bp male-specific amplicon (MSA). On the other hand, **emb01F16** was only useful for determining hybrid purity of one hybrid (Brinjal Asha) resulted in amplification of 150bp female- specific amplicon (FSA) as well as 170bp male-specific amplicon (MSA). Further, these co-dominant markers (**eme08D09** & **emb01F16**) was tested on all the four-sample size of each hybrids to detect the heterozygosity of the hybrids. In 400 hybrid sample size of Arka Anand, the percentage of hybrid purity in SSR analysis was calculated to be 95% (Table 2). Similarly, in the case of 300, 200 and 100 hybrid sample size of Arka Anand, the percentage

Table 2. Comparison of hybrid purity assessment based on GOT and SSR analysis with different sample size in Brinjal hybrids.				
Brinjal (Arka Anand)				
Sample size	GOT (%)	Putative false hybrid individuals	SSR Analysis (%)	Putative false hybrid individuals
400	95	20	95	20
300	95	15	94.7	16
200	95	10	95	10
100	95	5	95	5
Brinjal (Asha)				
Primer 1				
Sample size	GOT(%)	Putative false hybrid individuals	SSR Analysis (%)	Putative false hybrid individuals
400	95	20	95	20
300	95	15	94.3	17
200	95	10	95	10
100	95	5	95	5
Primer 2				
Sample size	GOT(%)	Putative false hybrid individuals	SSR Analysis (%)	Putative false hybrid individuals
400	95	20	95	20
300	95	15	94.3	17
200	95	10	95	10
100	95	5	95	5

of hybrid purity was calculated to be 94.7%, 95% and 95%, respectively (Table 2). In 400 hybrid sample size of Brinjal Asha, the percentage of hybrid purity in SSR analysis was calculated to be 95% (Table 2). Similarly, in the case of 300, 200 and 100 hybrid sample size of Brinjal Asha, the percentage of hybrid purity was calculated to be 94.3%, 95% and 95%, respectively (Table 2).

Based on the results obtained from present study, it is clear that there is a need to critically assess the hybrid purity of popular cultivars of premium quality at each and every stage of seed multiplication and processing with the help of molecular markers so that, seeds cultivated by farmers are true-to-type and fetches premium price. In the present study where, simultaneous laboratory and field study has been done for hybrid purity check of brinjal hybrids, that to in the past number of similar approaches gain promising results and eliminated any bias factor in laboratory analysis (Smith and

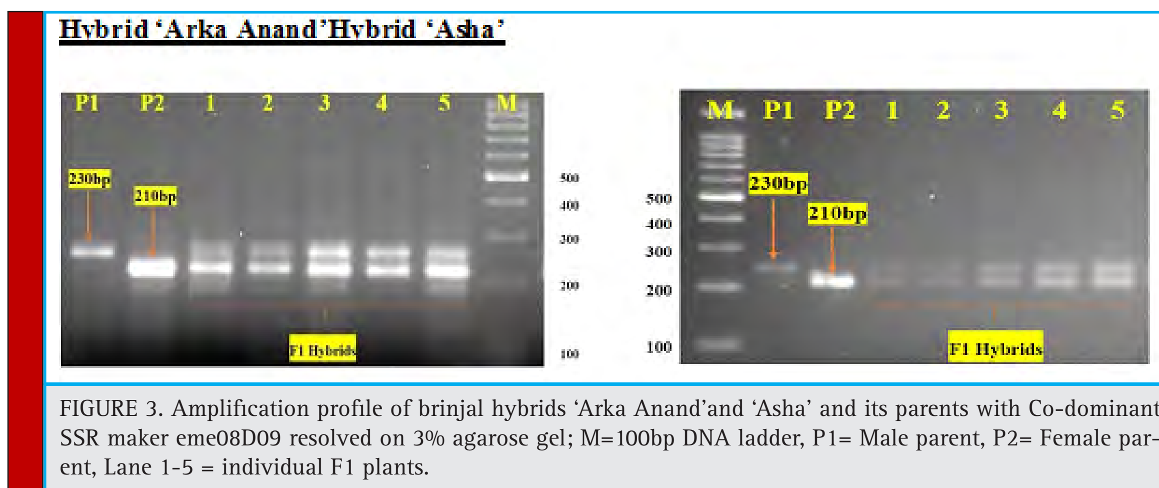

FIGURE 3. Amplification profile of brinjal hybrids 'Arka Anand'and 'Asha' and its parents with Co-dominant SSR maker eme08D09 resolved on 3% agarose gel; M=100bp DNA ladder, P1= Male parent, P2= Female parent, Lane 1-5 = individual F1 plants.

Table 3. List of polymorphic SSR markers used in the study.

Sl. no.	Primer Name	Primer Sequence 5' – 3'
1	emi04H10 F	ATCGGAGCAAGAGACATTAGATGC
	R	GTTTCCAACACAGTCCCCAATACAACAA
2	emb01D10 F	AAGAATCGGTCCTCTTTGCATTGT
	R	TGCTTTTCACCTCTCCGCTATCTC
3	**eme08D09 F**	**ATGGATTAGCATGTGGAGGACTGAA**
	R	**GTTTCATGGTAGGTGGAGACAGAACCA**
4	emf21K08 F	ATCAATGACACCCAAAACCCATTT
	R	GTTTGAAAACCCAATACAAATCCGA
5	emg11M21 F	ATAGCCTACTGCCTTCAAGACCAT
	R	GTTTCCTACGTCCAGTCCCCTTAGGT
6	emb01J19 F	GACAGGGATAGGGGTACGGATAGG
	R	ATCCATGTGATGCCTCGATTTTCT
7	**emb01F16 F**	**AAAACAGAAGCAAAGTCGGCAGTC**
	R	**GTCCACCAACACCTTACCATCCTC**
8	emi06A04 F	ATTTGGGACAAATGTGGGTGAGAC
	R	GTTTCCACGCTACTTAGGGGACTCAA
9	eme03F04 F	ATATGACGACAGACGTAAAGCGACC
	R	GTTTCAGAGTTTTGCCATCTGTGTCGAG
10	emf11B07a F	ACGAGAGTTGCTACAGTTAAGGGG
	R	GTTTGGGGACCAAAGTGTATTTTCAAGG
11	emk04H07 F	ATTTGGCTGGGTTGTTGGTCTAGT
	R	GTTTGGCCCAATTACTCAAATACCCTG
12	emf21C11 F	AGGTTGGAGCCATGATTACTTGAA
	R	GTTTGCTACCTATCAAACAGGCGGAA
13	emf01O04 F	ATCCGTTGATACTAGCCGTTGCCT
	R	GTTTCACCCGGTATGAGTGTATCCC
14	emk04N11 F	ATCTCCCCCTCAACTTTGAACAAT
	R	GTTTGTGTGATATAGCCCAACAATTCAC
15	emh11N11 F	ATTCAGTTCTTCGCTTTGGAGCTT
	R	GTTTCCAAACCCGACCCATCCTAAATAA
16	eme05G05 F	ACAAGAAAGAGGAGCTGGGGAAATTG
	R	GTTTCCTTCTTGGGAAGACAACTTATCA
17	eme01D03 F	ACAAGAATCGGTCCTCTTTGCATTGT
	R	GTTTGCTTTTCACCTCTCCGCTATCTC
18	emg11P03 F	ACTCGCCTCTCTCAATCTTTCTTG
	R	GTTTCAATATAACCTCGGCTATGAGACCC
19	emd01C04 F	ACCTGCATGAAATGTGTTTGAGTG
	R	GTTTGGGTCTTTTCATCTCAAAATGGG
20	emh05H12 F	AGTCACTGCTCTTAGTTTCTGCAA
	R	GTTTCAGAGCAGCGATCCTTTCTTCATT
21	emd01A01 F	ACAGCAAAATGCCTAATGACAGCACA
	R	GTTTATGCCTGACTCTGCTTGTGCCTA

Wych, 1986; Orman et al., 1991). As Singh and Singhal (1999); Tunwar and Singh (1988) recommended to use minimum 400 individual seeds for purity testing as per Indian minimum seed certification standards, we have observed that by using SSR primers (eme08D09 and/or emb01F16) with only 100 seeds sample size; hybrid purity could be calculated upto 95% percentage which was in agreement with the results obtained via, 400 sample sizes tested in GOT. Hence results indicated that instead of handling 400 samples in GOT, use of only 100 seeds in SSR analysis proving useful with less time and cost concerned approach.

The present study also recorded the same results with SSR markers when tested with sample size of 400, 300 and 200. Hence instead of using 70 days long protocol involved with GOT, by applying few days protocol of SSR with the given primers; it will assure the early releases of hybrid seeds. As per study results of field grow out tests and SSR markers of individual seeds; highly significant correlation does exist between two studies and more reliability to the latter technique. As the results were fairly consistent; hence SSR markers study can be recommended as a supplementary technique for making speedy decision to accept or reject hybrid seed lots based on contamination.

GOT certainly delays the whole process of decision making, packaging and marketing of the commercial seed. Thus, farmers did not get the hybrid seeds at right time for sowing, resultant precluding the immediate cultivation of the hybrid seed produced. In addition to capital invested on hybrid seed production and additional expenditure incurred on storage of hybrid seed, GOT ultimately increases the hybrid seed cost (Nandakumar *et al*, 2004). Similar to present study, use of SSR markers for genetic purity testing has been demonstrated in rice (Nandakumar et al., 2004); in maize (Wang et al., 2002) and in sunflower (Pallavi et al., 2011) and overall data remained comparable with field grow out test. Similar to SSR marker applied in present study with minimum sample size; number of workers with other plants have applied different sample size of seeds for RAPD primers for example: 400 seeds in chicory (Bellamy et al., 1998), 120 in canola (Marshall et al., 1994), 40 in tomato (Rom et al., 1995), 30 in Chinese cabbage (Meng et al., 1998) and 10-20 in pepper (Ballester and Vicente, 1998) to assess the genetic purity of the hybrid seeds.

Overall data of the present study have showcased that SSR markers are quick, reliable and results are mostly consistent with morphological analysis in the field study. Markers reported in the study could be involved for routine genetic purity testing of Arka Anand and Brinjal Asha hybrids. The SSR markers developed through this study will be useful for seed industry to select appropriate marker combinations and assess genetic purity of the crop.

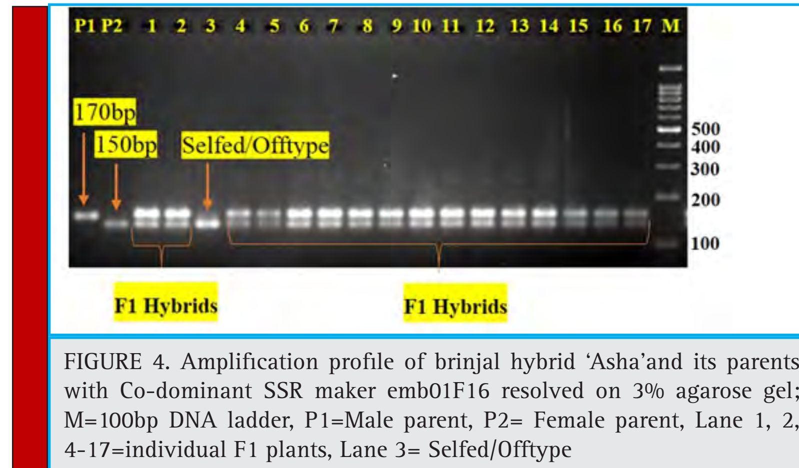

FIGURE 4. Amplification profile of brinjal hybrid 'Asha'and its parents with Co-dominant SSR maker emb01F16 resolved on 3% agarose gel; M=100bp DNA ladder, P1=Male parent, P2= Female parent, Lane 1, 2, 4-17=individual F1 plants, Lane 3= Selfed/Offtype

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Biodegradation of cotton seed soapstocks by novel indigenous *Bacillus species*

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ABSTRACT

Soapstocks is a value-added by-product separated from vegetable oil refining operations. *Bacillus sp.* is a good enzyme producer. The present research paper focused at isolation, screening and identification of *Bacillus sp.* from soapstock samples. Cotton seed soapstock samples used in the study were 7% oil rich gelatinous dark brown chemical compound, which were enriched, serially diluted and spread on tributyrin agar plates, to isolate lipase positive cultures. Qualitative analysis of lipase producing microorganisms was done by plate assay on tributyrin agar plate and zone of hydrolysis measured. *Bacillus sp.* were further screened for cellulase and protease production by plate assay. Two cultures were identified on the basis of molecular and biochemical characteristics as *Bacillus licheniformis* (3B) and *Bacillus pumilus* (18B). Among selected *Bacillus cultures Bacillus licheniformis* and *Bacillus pumilus* gave-good zone of lipase and cellulasehydrolysis. Bacillus pumilus was highly protease producing organism. Quantitative analysis of Lipase production activity measured spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate. *Bacillus licheniformis* showed 1.72 U/ml lipase productions whereas *Bacillus pumilus* (18B) has 2.59 U/ml. Phylogenetic trees showed similarity with other highly similar species.

KEY WORDS: *BACILLUS LICHENIFORMIS*, *BACILLUS PUMILUS*, LIPASE, P-NPP, PHYLOGENETIC TREES

INTRODUCTION

Industrial wastes, vegetable oil processing factories, soil contaminated with oil etc contain oily environment which provides a good environments for lipase producing microorganisms (Vandana *et al.,* 2014). Soapstocks is a gelatinous dark brown undesirable chemical compound product from vegetable oil refining operations (King *et al.,* 1998). Crude oil contamination in the environment has lots of hazard and so remediation of crude oil creates area of interest for research (Guru *et al.,* 2013). Microbes secrete various enzymes among them lipase which helps

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in degradation of oil (Veerabagu *et al.,* 2014). Research in bacterial lipases is of great demand now because of value added potential industrial application (Sirisha *et al.,* 2010). Industries are seeking lipase producing strains of bacteria which contain excellent properties using cost effective methods on large scale production (Bharathi *et al.*, 2018).

Lipase (triacyl glycerol acylhydrolases, EC 3.1.1.3) catalyzes the hydrolysis of triacyl glycerol to glycerol and long chain fatty acids at oil water interface (Pualsa *et al.,* 2013). Research can be done toward lipases from plant and animal origin but lipases from microbial origin are receiving much attention with the rapid development of enzyme technology. Lipase act as biocatalysts constitute the significant important role for biotechnological applications (Hasan *et al.,* 2006, Saxena *et al.,* 1999). Microbial lipases constitute much application such as in the detergent industry, food industry, paper and pulp industry, organic synthesis, bioconversion in aqueous media, resolution of racemic acids and alcohols, regioselectiveacylations, ester synthesis, oleochemical industry and lipases in medical application (Sharma *et al.,* 2001, Verma *et al.,* 2012, Mauti *et al.,* 2016, Saraswat *et al.,* 2017)

This study was conducted to isolate lipase producing bacteria which were screened on tributyrin agar plates. They were further analyzed for cellulase and protease production by plate assay. The bacterial genus *Bacillus* were identified on the basis of biochemical tests and molecular 16s r DNA Partial Gene sequencing analyzes. Quantitative analysis of lipase production was done spectrophotometrically using p-NPP as substrate. Further study will conducted on enzymatic degradation.

MATERIAL AND METHODS

Soapstock samples were collected from two different cotton oil refinery industries nearby Kadi (North Gujarat), India. At the starting season of cotton (November), Soapstock samples were collected from the flowing stock at Washer discharge end of the pipe in a sterile and air tight container. B/H (Bushnell–Haas) medium was selected for enrichment of cotton seed oil soapstocks for microbial growth (Guru *et al*., 2013).10 gram of cotton seed oil soapstock samples were added to 100 ml of B/H mediums and incubated at 37°C in static condition for 5 days. From each sample, 1ml of enriched samples were transferred to the 100 ml of Tributyrin broth medium incubated at 37 °C, in shaking condition at 100rpm for 48 hours. Enrichment was performed over a 7 days of incubation.Enriched Soapstock samples were serially diluted. Diluted samples were spread on to Tributyrin agar medium for isolation of Bacteria .TBA Plates were incubated at 37°C for 2days. Isolated colonies were purified on same medium by streak plate method. Pure cultures isolate were preserved at low temperature in Nutrient agar slants for screening and further use. Lipase-producing strains were screened by qualitative plate assay according to Lokre *et al.,* 2014. Isolates were spot inoculated on tributyrin agar plates and incubated at 37°C for 2 days. Zone of clearance was observed due to hydrolysis of tributyrin by lipase enzyme.

Cellulase and Protease activity were done by qualitative agar plate assay in nutrient agar media containing respective substrates. Culture was spot inoculated and incubated at 37 °C for 2 days. Check for the zone of clearance around the colonies due to utilization of the particular substrate.

Culture was grown in medium containing 1% carboxy methyl cellulose (Dabhi *et al.,* 2014). After incubation the CMC plates were flooded with 0.1 % congo red staining, after 5 min stain was discarded and the plates were distained by 1M NaCl solution with continuous stirring for 15-20 min. The clear zone around colonies indicated cellulose hydrolysis.Protease activity was checked in medium containing 1% skim milk as substrate (Prabavathi *et al.,* 2012). Spot inoculated cultures were incubated at 37 °C for 2 days and observed for clearance zone around colonies.

Selected Bacterial cultures that show Positive lipase production in plate assay, which were subjected further for Quantitative estimation. 2 days old bacterial cultures grown on TBA medium were used for inoculation. One loopfull culture was inoculated into 100 ml of inoculum medium containing: peptone 0.5%, Yeast extract 0.5%, NaCl 0.5% and cotton seed Oil 1%. Cultures were incubated at 37°C and 100 rpm for 4hrs. 5% inoculum medium was further inoculated into 100 ml of same medium (as mentioned above) for lipase production and incubated at 37°C and 100 rpm for 5 days. Enzyme assay was performed according to the method by Winkler *et al.,* 1997 with some modification. The culture filtrate (production medium) was removed at 24 hr interval from each flask & centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was used for enzyme assay. Lipase activity was determined by a spectrophotometric assay using p-nitrophenyl palmitate (pNPP) as substrate. P-NPP was hydrolysed by lipase to give p-NP which gave yellow color, absorbance of which was measured spectrophotometrically at 410 nm against enzyme free blank. Statistical Analysis were done in Microsoft word excel data analysis of lipase production.

The isolates showing maximum zone of clearance were selected for further analysis. Morphological and biochemical characteristics of the isolates were studied for the identification of the potent Bacterial isolate. Molecular characterization of potent Bacterial strains

was done by 16s rDNA partial Gene sequencing analysis. It was carried out at Biogene department of GSBTM, Gandhinagar.

The bacterial isolates were identified on the basis of their morphological characteristics (like cell shape, Gram staining, spore staining and motility) and biochemical tests viz. According to Cappuccino *et al.*,1996 biochemical test were done like Voges Proskaurtes test, Citrate utilization, Gelatin hydrolysis, Nitrate reduction, Ornithine decarboxylase, Lysine decarboxylase, Catalase test and hydrolysis, Indole test, Starch hydrolysis, H2S production, and Gas production from glucose. The utilization of different sugars was studied using bacterial identification kit from HiMedia.

MOLECULAR IDENTIFICATION USING 16S RDNA SEQUENCING

Two bacterial isolates (3B and 18B) were identified using 16S rDNA sequencing. DNA was isolated from these bacterial isolates and its quality was evaluated on 1.2% agarose gel. The 16S rDNA gene was amplified by PCR from the above isolated DNA and the PCR amplicon was purified to remove contaminants. 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nr database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

RESULTS AND DISCUSSION

SCREENING AND ISOLATION OF LIPOLYTIC BACTERIA

From enriched Soapstock samples, total 49 pure cultures were isolated. Among them 30 cultures were bacterial isolates. All 30 bacterial isolates were lipase positive, 6 bacterial cultures were protease producers and 10 bacterial cultures were cellulase producers. Best two highly positive cultures were selected on the basis of qualitative analysis of lipase, cellulase and protease by plate assay as shown in Table 1 and Fig 1. In 2014 Ali et al, reported that the lipolytic bacterial Spp. isolated from oil contaminated soil were dominantly from genus Bacillus and Psudomonas with 23% percentage of occurrence of *Bacillus spp.* among different bacteria in samples, fol-

Table.1 Measures of Clear zone diameter to colony diameter ratio of bacterial isolates.

Sr. no	Bacterial Isolate	Clear Zone Diameter	Colony Diameter	Ratio
Lipase activity				
1	*Bacillus licheniformis* (3B)	20 mm	15 mm	1.33 mm
2	*Bacillus pumilus* (18B)	22 mm	16 mm	1.37 mm
Cellulase activity				
1	*Bacillus licheniformis* (3B)	9 mm	5 mm	1.80 mm
2	*Bacillus pumilus* (18B)	9 mm	5 mm	1.80 mm
Protease activity				
1	*Bacillus licheniformis* (3B)	30 mm	16 mm	1.87 mm
2	*Bacillus pumilus* (18B)	31 mm	10 mm	3.10 mm

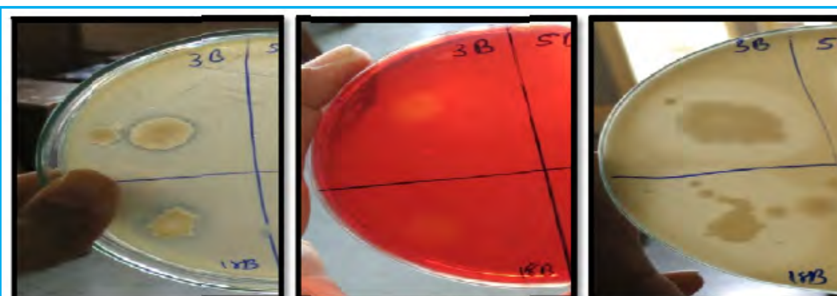

FIGURE 1. A. Lipase positive cultures on 1% tributyrin agar plate. B. Cellulase positive cultures on 1% CMC agar plate. C. Protease positive cultures on 1% Skim milk agar plate

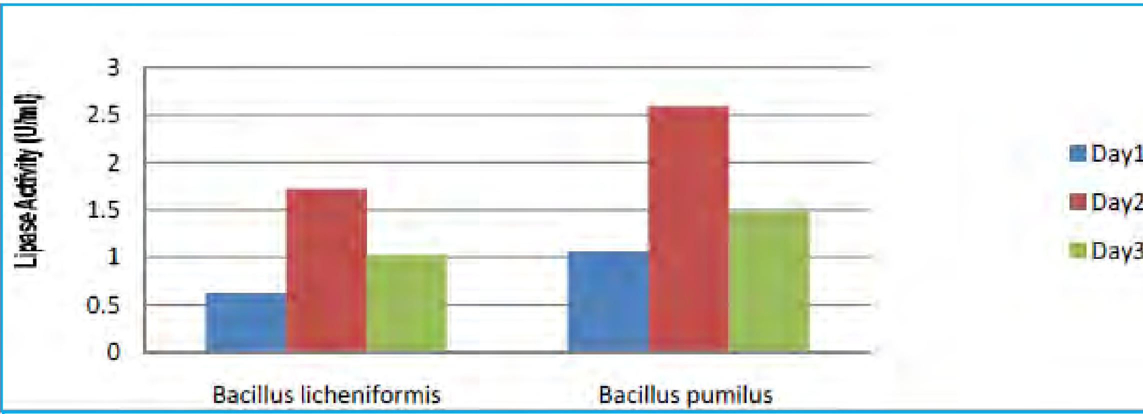

FIGURE 2. Lipase production of bacterial isolates in U/ml/min.

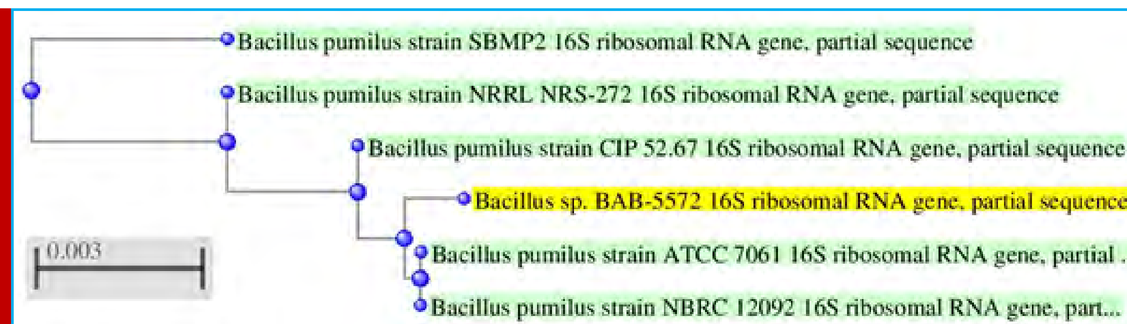

FIGURE 3. Phylogenetic tree of 18B *Bacillus pumilus*

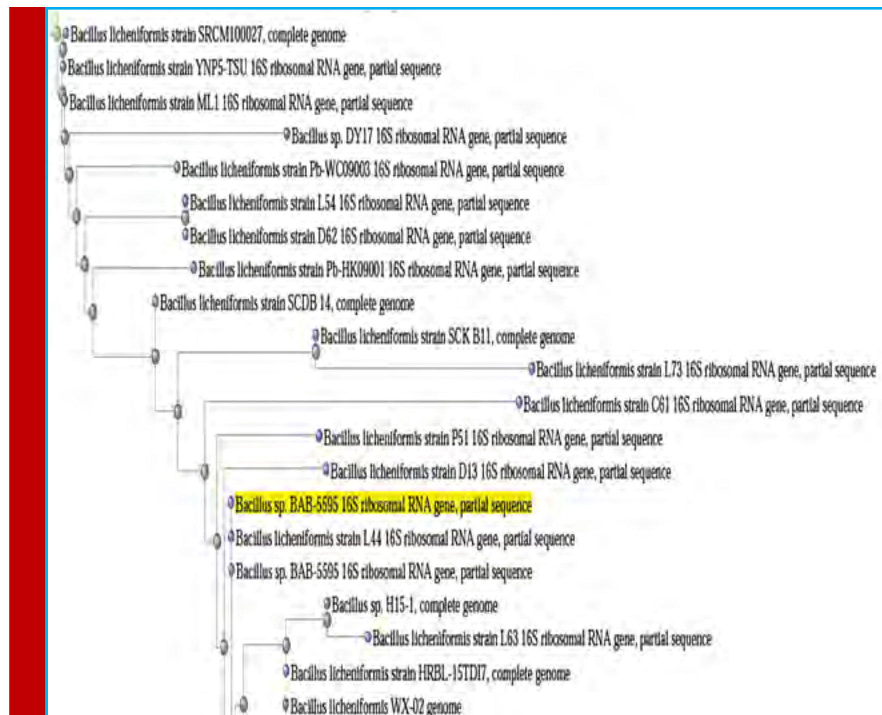

FIGURE 4. Phylogenetic tree of 18B *Bacillus licheniformis.*

Table 3. Morphological and Biochemical test of isolated bacteria

Sr. no	Biochemical test	*Bacillus licheniformis* (3B)	*Bacillus pumilus* (18B)
1	Gram's staining	Gram positive	Gram positive
2	Motility	+	+
3	Endospore	-	-
4	Methyl red	+	+
5	VogesProskauer's	+	+
6	Citrate Utiliation	+	+
7	Indole	+	+
8	Glucuronidase	+	+
9	Nitrate reduction	+	+
10	PYR	+	+
11	ONPG	+	+
12	Lysine utilization	+	+
13	Esculin hydrolysis	+	+
14	Arginine utilization	+	+
15	Lactose	-	-
16	Xylose	-	-
17	Maltose	+	-
18	Fructose	+	+
19	Dextrose	+	+
20	Galactose	+	+
21	Raffinose	-	-
22	Trehalose	+	+
23	Melibiose	-	-
24	Sucrose	+	+
25	L-Arabinose	+	+
26	Mannose	+	+
27	Inulin	+	+/-
28	Sodium gluconate	+	+
29	Glycerol	+	+
30	Salicin	+	+
31	Dulcitol	+	+
32	Inositol	+	+
33	Sorbitol	+	+/-
34	Mannitol	+	+
35	Adonitol	-	+
36	Arabitol	-	+
37	Erythritol	+	+
38	alpha-Methyl-D-glucoside	+	+
39	Rhamnose	+	-
40	Cellobiose	+	+
41	Melezitose	-	+
42	alpha-Methyl-D-Mannoside	+	-
43	Xylitol	-	+
44	ONPG	-	+
45	Esculin	-	+
46	D-Arabinose	+	+
47	Citrate utiliation	-	+
48	Malonate	-	-
49	Sorbose	+	+

lowed by Pseudomonas spp. 18%. Bacillus sp. has been potential for production of proteases and lipases (Sangeetha *et al*., 2010). Bacterial *Bacillus species* are the prominent source of lipases in which *B. subtilis* (Shah et al., 2006), *Bacillus pumilus* (Sangeetha et al., 2008), *Bacillus licheniformis* (Madan et al., 2009, Sangeetha et al., 2010) are potent strains for lipase enzyme production. For the enzyme production we have done plate assay with various enzymes like lipase, cellulase, protease from which *B.pumulis* gives maximum zone of hydrolysis of 1.37 mm with lipase and 3.10mm with protease while Bacillus licheniformis and *Bacillus pumulis* gives same maximum zone of hydrolysis of 1.80 mm with cellulase. From the above isolated organisms, *Bacillus pumilus*, *Bacillus licheniformis* isolates were found to be true lipase, protease and cellulase producers giving maximum zone of hydrolysis. Further screening was done for various enzymes. The isolate 3B and 18B which further quantitatively tested for production of crude lipase by pNPP as assay substrate and are efficient to produce 1.72 U/ml and 2.59 U/ml of crude lipase enzyme respectively depicted in figure 2.

In Statistical Analysis p- value analyzes for control, 3B and 18B lipase productions were <0.0016, <0.024 and <0.020 respectively. *Bacillus pumilus* is the best possible isolate having highest lipase production and was further screened and optimized for lipase production. The maximum amount of lipase production was obtained on the day 2nd with recoverable enzyme activity gradually decreasing thereafter in shaking conditions. Consequently, further studies were carried out on cultures incubated for 3 days to obtain enzyme production. Biomass production remained stable, after which the culture reached the stationary phase. This forces microbes to produce enzymes to degrade crude oil to utilize it as a source of energy and these enzymes which were capable of producing certain secondary metabolites (Guru *et al*., 2013, Adnan *et al*., 2018) *P. gessardi* was a novel lipase degrading bacteria from the oil spilled soil which can be useful for the remediation of oil contaminated soil. (Veerabagu *et al.*, 2014), *Pseudomonas synxantha PS1* a lipase producing strain from oil well produced water having strong biodegradabitility of waste grease (Cai *et al.*, 2016) as well as fungi aspergillus nigar able to degrade petroleum hydrocarbon (Mauti *et al.*, 2016)

It can be concluded that from the results of the present study that *Bacillus pumilus* could be used as new potent microbial source of lipase. In further studies pilot scale production and purification studies will be conducted.

CHARACTERIZATION OF BACTERIAL ISOLATES

The bacterial isolates which showed maximum zone of clearance for lipase production were subjected to further characterization and identification by morphological, biochemical and molecular (by 16s rDNA sequencing) characteristics. The culture code 3B known as *Bacillus licheniformis* with *a*ccession Number KU728636 and 18B known as known as Bacillus *pumilus* with accession Number KU728634. Phylogenetic trees are show in Fig. 3 and 4.

From the table-2 we conclude from microscopic, morphological, cultural characteristics and biochemical studies that the organism is gram positive rod, aerobic, mesophilic, highly mobile, non-endospore former, lactose non fermenting *Bacillussp*..Based on its morphological and physiological characteristics, the isolates were given for 16s r RNA and it was confirmed that they belong to Bacillus genus.

CONCLUSION

Screening for lipase producing cultures from cotton oil refinery industries and resulted in the isolation of 49 isolates including bacteria & fungi.The isolate which showed highest production of lipase in plate assays were further quantitatively tested for production of lipase by pNPP as substrate assay. The culture was identified by morphological and molecular basis as *Bacillus licheniformis* & *Bacillus pumilus*. Culture was deposited in the NCBI culture collection center with accession number. Presence of cellulase and protease enzyme may help in degradation study.

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Tap water quality assessment of some selected regions of Mhow, District Indore India

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ABSTRACT

An attempt has been made to analyse the physicochemical and bacteriological examination of ground water (Tap water) seasonally (rainy, winter and summer seasons) at selected regions of Mhow Tehsil area (Sangi Street, Raj Mohalla, Kali Mata Mandir area, Cantonment Board area and Main Street) for two years, during 2011-2013. During physicochemical examination, Water Colour, Temperature (°C), pH values, Total hardness (mg/lit), Specific conductivity (µmhos/cm), Total alkalinity (mg/lit), TDS (mg/lit), Chloride (mg/lit), Fluoride (mg/lit), Nitrate (mg/lit), Phosphate (mg/lit), Sulphate (mg/lit) and BOD (mg/lit), DO (mg/lit) and COD (mg/lit) values have been analyzed however, the total coliforms (MPN/100ml) and faecal coliforms (MPN/100ml) were also estimated during bacteriological examination. Continuous monitoring and environment management programs should be run properly to manage the elements in limit range which is necessary to control drinking water pollution.

KEY WORDS: TAP WATER, MHOW TEHSIL AREA, PHYSICOCHEMICAL AND BACTERIOLOGICAL EXAMINATION

INTRODUCTION

Ground water plays an important role in our environment and our economics. In the environment it supports rivers, lakes, wetlands, springs, ponds, marshland, swamps, streams and used as an important sources of freshwater around the world. Groundwater is available at purest form in nature which is colourless and tasteless. Safe potable water is not likely to be harm humans which keeps healthy lives throughout the world. The quality of ground water depends on various physiochemical constitutes and their geographical data of the particular region. Ground water is the major sources of drinking water. The clean drinking water is one of the essential compounds that profoundly influence life. The deficiency of the clean water increases day by day due to

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pollution of water. In recent years, an increasing threat to ground water quality is the results of human activity at ground water. Now water is getting contaminated, which can cause water borne diseases and gets health hazards, (Shahida and Ummatul, 2015, Singh 2016, Jena and Sinha, 2017, Nagaraju *et al.*, 2018 and Patil 2018).

It has been reported that the more than 90% of population of various states in our country are dependent upon groundwater source for drinking and other purposes (Ramachandraiah, 2004; Tank, 2010). Now days, groundwater is used in agricultural and industrial sector (Ramesh and Soorya, 2012). However, due to increase in industrialization and urbanization, deterioration in the quality of groundwater has been noticed (Laluraj *et al.*, 2005 Leelavathi_*et al.*, 2016; Prajapati and Rokde, 2016; Behailu *et al.*, 2017; Soni and Singh, 2018).

The availability of pure water through surface and groundwater resources has become more critical day today. Only 1% of surface and groundwater resources are available on earth for drinking purpose, domestic purpose, power generation, industrial consumption, agricultural purpose, transportation and waste disposal (Mishra *et al.*, 2002; Tahir, *et al.*, 2008). The majority of the recent problems related to drinking water contamination, associated with pollution of surface and ground water resources and with the formation of reaction by-products resulting from the use of disinfectants and oxidants in drinking water treatment, are closely connected with the rapid advances in analytical techniques. It has been noticed that the most common and wide spread danger associated with drinking water is the direct or indirect contamination by sewage, human and agriculture, chemical and industrial influents etc. (Clark *et.al.*, 1982 and WHO, 1985). It is therefore considering the importance of health problem in context of ground water (Tap water) contamination.

Mhow is that the cantonment of the Indore district in M.P. The water from the main source is the municipal corporation supply to fulfil the need of population. Thus quality of potable and non potable Tap water resource of Mhow is an urgent need to protect human health. Hence, it was thought to study physico-chemical and bacteriological parameters of different Tap water resources of Mhow. Therefore a continuous periodical monitoring of water quality is necessary. In order to protect human health from different water borne and water related diseases appropriate steps should be taken. The study will be helpful in the management of water resources and save human health of Mhow Tehsil from environmental pollution.

Although various workers have contributed in the field of water monitoring related to health problems in various parts of India, yet most of the parts of Madhya Pradesh are neglected even today. Mhow is a cantonment of Indore, but there is no work done in this area. Therefore, looking to the importance from health point of view, the present work has been aimed to analysis of potable or non-potable Tap water resources of Mhow Tehsil. The undertaken work has been aimed to study the physicochemical and bacteriological examination of ground water (Tap water) seasonally (rainy, winter and summer seasons) at selected regions of Mhow Tehsil area (Sangi Street, Raj Mohalla, Kali Mata Mandir area, Cantonment Board area and Main Street) during 2011-2013.

MATERIALS AND METHODS

The physicochemical and bacteriological examinations of ground water (Tap water) were analyzed seasonally (rainy, winter and summer seasons) as per standard methods of APHA (2005). The samples were collected seasonally (Winter–December/January, Rainy–July/August, Summer–April/May) from the various sampling station in the morning (between 8 a.m. to 11 a.m.) for the consecutive two year (2011- 2012 and 2012 -2013). For the analysis of physicochemical parameter of ground water (Tap water) APHA, 2005 was applied.

- Hydrogen ion concentration (pH): The hydrogen ion activity of the water sample was measured with the help of calibrated analyzer (pH meter).
- Colour: Colour of water was observed by visual comparison method.
- Temperature: The water samples were collected in suitable container, measured with the help of mercury thermometer.
- Specific conductivity: It was measured with the help of a Conductivity meter. The unit of conductivity measurement is **μmhos/cm**.
- Dissolved oxygen: Dissolved Oxygen was measured titrimetrically by Winkler's method
- Biochemical oxygen demand: Biochemical oxygen demand was estimated by 5 days BOD method (APHA, 2005).
- Chemical oxygen demand: Chemical oxygen demand was estimated through titrimetric method
- Alkalinity: Total Alkalinity was estimated by titrimetric method
- Sulphate: Sulphate was estimated by gravimetric method.
- Phosphate: Phosphate was measured by spectrometric method
- Nitrate: Nitrate was measured by spectrometric method
- Total Hardness: Hardness was the combination of Ca or Mg ions. The total hardness was estimated by titrimetric method

- Chloride: Chloride was estimated by argentometric method
- Fluoride: Fluoride was estimated by SPADNS calorimetric method
- Methods for analysis of bacteriological parameter

Bacteriological parameters of ground water (Tap water) were also analysed as per standard methods of APHA (2005).Total coliform microbes (Bacteria) - Total coliform in ground water were estimated by multiple tube method. Results were obtained in MPN (Most Probable Number) per 100 ml by consulting the MPN table.Faecal coliform microbes (Bacteria) - Faecal coliform were also estimated by multiple tube method by using BGB broth. Results were also documented in MPN (Most Probable Number) per 100 ml by consulting the Most Probable Number table.

RESULTS AND DISCUSSION

The results obtained in present investigation have been summarized by Figures (1-5). The true colour has been noticed for tap water around all the studied area during rainy, winter and summer seasons during 2011-2013. The highest and the lowest temperature (°C) were recorded as 40°C (Summer, 2012 -2013) for Main street and 13 ^{o}C (Winter, 2011-12) for Sangi Street, respectively. The pH values were noticed the lowest as 7.1 (Rainy, 2011 -2012) for Main street and the highest as 8.1 (Summer, 2012-13) for Main street also. Whereas, the total hardness (mg/lit) were estimated the lowest as 100 mg/lit (Rainy, 2011-12, for Sangi Street and Raj Mohalla both) and this value were analysed the highest as 139 mg/ lit (Summer, 2012-13, for Cantonment Board area). Specific conductivity (µmhos/cm) value were noticed the lowest and the highest as 176 µmhos/cm (Rainy, 2011 -2012) for Sangi Street and 412 µmhos/cm ((Rainy, 2012-13) for Kali Mata Mandir area, respectively. Total alkalinity (mg/ lit) value were found to be the lowest as 74 mg/ lit (Rainy, 2012-13, for Raj Mohalla both) and the highest as 110 mg/ lit (Summer, 2011 -2012 for Kali Mata Mandir area and Main street both).

The values of TDS(mg/ lit) were noticed highest as 289 mg/ lit (Rainy, 2012-13, for Kali Mata Mandir area) and lowest as 158 mg/ lit (Winter, 2011-12, for Cantonment Board area).Whereas, Chloride (mg/ lit) value has been calculated the lowest as 120 mg/ lit (Rainy, 2011 -2012 for Kali Mata Mandir area) and the highest as 230mg/ lit (Summer, 2011 -2012, for Cantonment Board area). Fluoride (mg/lit), Nitrate (mg/lit), Phosphate (mg/ lit) and BOD (mg/lit) value were fluctuated in the range of 0.1 -0.6, 1.1-1.7, 0.01-0.02 and 1.0-2.0 respectively during studied season of experimental period. The value of Sulphate (mg/lit) were found the highest as 82mg/lit (Rainy, 2011-2012) for Sangi Street and the lowest as 19mg/lit (Summer, 2011-12) for Main street.

The DO(mg/lit) contents were found to be in the ranged between 6.1 mg/lit (Winter, 2011-12, for Cantonment Board area) to 7.9 mg/lit (Summer, 2012-13, for Main street). Whereas, COD (mg/lit) value were also noticed the highest as 10.4 mg/lit(Rainy, 2012-13, for Cantonment Board area) and the lowest as 7.2 mg/lit (Winter, 2011-12, for Raj Mohalla). However, the total coliform (MPN/100ml) were found 2 to 4 (Sangi Street and Raj Mohalla area) during rainy seasons only but faecal coliform (MPN/100ml) were found to be nil throughout the studied season of experimental period.

Analysis of groundwater quality is unavoidable because its poor quality may badly affect its users (Prasanna *et al.,* 2010). Various agencies like industrial effluents, agricultural runoff, sewage contributes several kinds of pollutants and nutrients in to the water bodies that brings about a series of changes in the physicochemical characteristics of water, which becomes the need of several investigations (Mahananda *et al.,* 2010). Whereas, water quality parameters has been assessed in Veeranna Cheruvu, Hasnapur, Mahabubnagar District, Telanagana State. It was found that the water quality parameters were within the permissible limits of standards and during the study period it has been noticed that many water quality parameters were minimum in monsoon and maximum in pre monsoon periods (Nagaraju *et al*., 2018).

It has been reported that the lowest and highest values of the borehole and spring water samples such as pH ranged between 6.34–6.37 and 6.34–7.92, EC between 627.33–621 µmho/cm and 566.33–569 µmho/cm, Total dissolve solids between 407.67–414.33 and 355.33–351.67 mg/lit and Total suspended solids between 14.37–14.83 and 13.00–13.08 mg/lit, respectively. Total hardness (TH), both calcium and magnesium hardness in terms of calcium carbonate concentration, ranged between 63.63–66.61 and 32.44–38.76 mg/lit respectively. Whereas, the highest and lowest concentration of NO2 were ranged between 0.11–0.12 and 0.05–0.06 mg/ lit, NO3 between 1.10–1.89 and 2.83–8.40 mg/lit, SO4 2- between 26.33–33.00 and 17.00–18.33 mg/lit and PO4 3- between 0.21–0.30 and 0.16–0.22 mg/lit in the borehole and spring water respectively (Shigut *et al*., 2017).

However, assessment of Water Quality Index (WQI) of groundwater in Rajkot District, Gujarat were done and noticed that the pH values ranged between 7.38-8.27 indicates that samples was changes from neutral to slightly alkaline. The TDS varied from 309-4858 mg/ lit, chloride concentration ranged from 57-2237 mg/ lit, total hardness ranged from 127 to 1582.40 mg/lit, Sulphate concentration ranged from 3-120 mg/lit and nitrate concentration in groundwater samples ranged

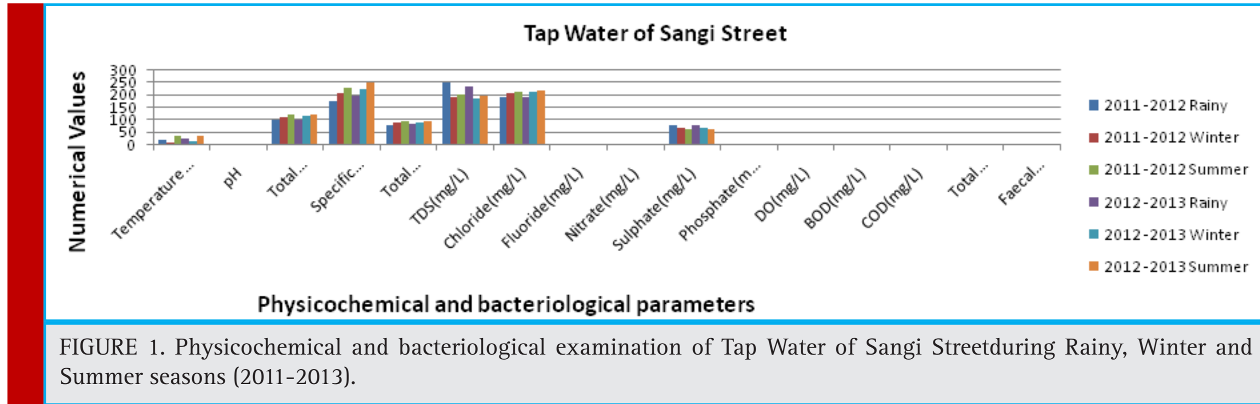

FIGURE 1. Physicochemical and bacteriological examination of Tap Water of Sangi Streetduring Rainy, Winter and Summer seasons (2011-2013).

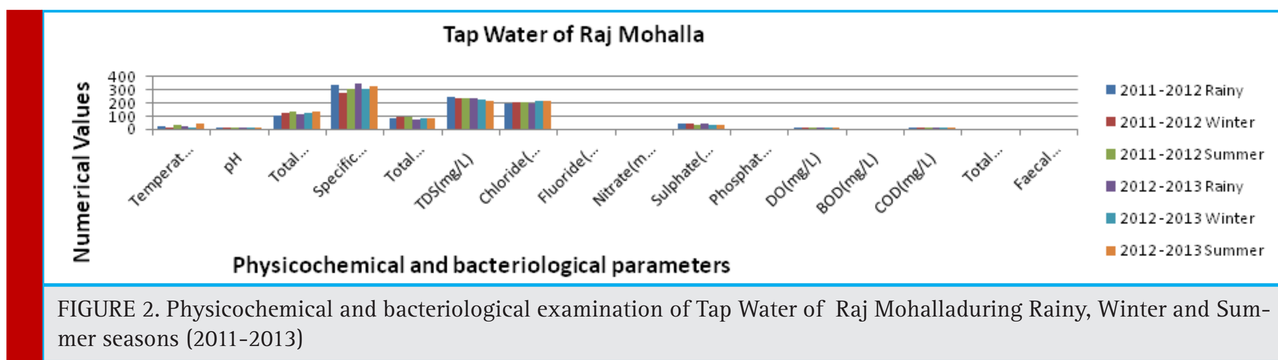

FIGURE 2. Physicochemical and bacteriological examination of Tap Water of Raj Mohalladuring Rainy, Winter and Summer seasons (2011-2013)

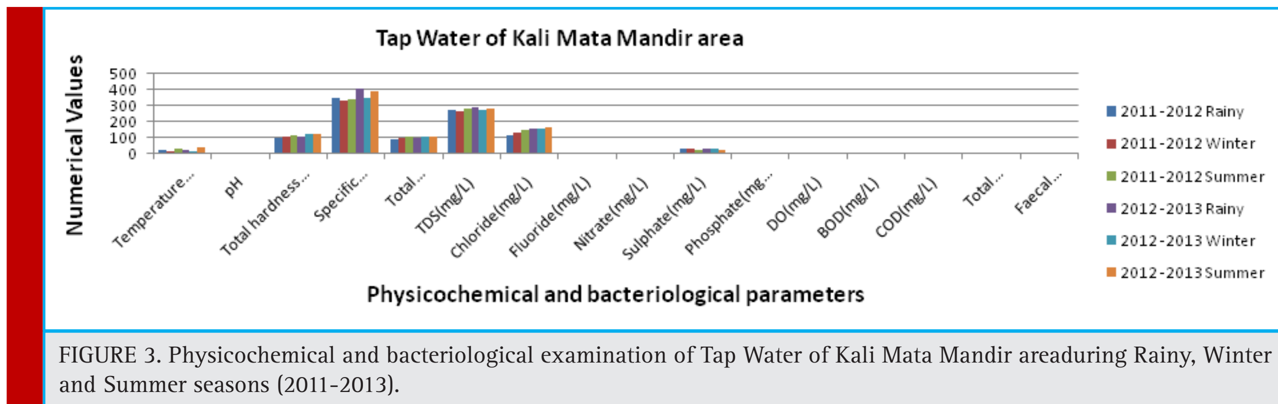

FIGURE 3. Physicochemical and bacteriological examination of Tap Water of Kali Mata Mandir areaduring Rainy, Winter and Summer seasons (2011-2013).

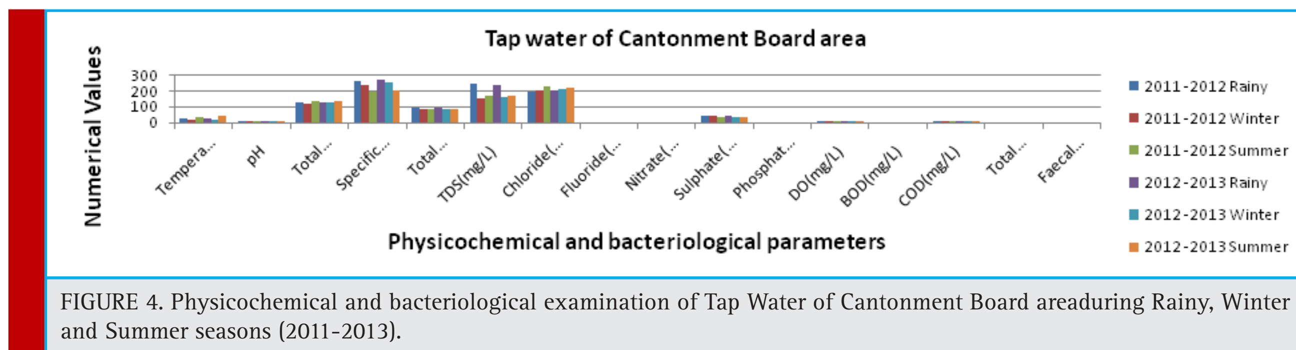

FIGURE 4. Physicochemical and bacteriological examination of Tap Water of Cantonment Board areaduring Rainy, Winter and Summer seasons (2011-2013).

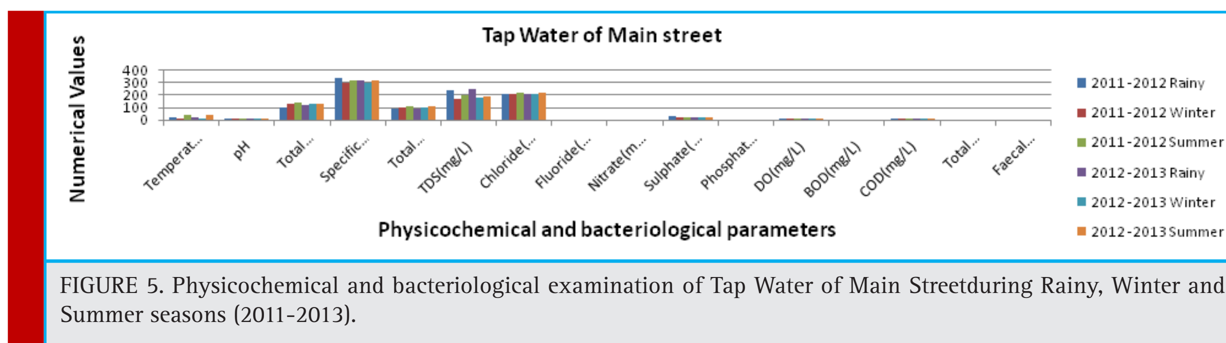

FIGURE 5. Physicochemical and bacteriological examination of Tap Water of Main Streetduring Rainy, Winter and Summer seasons (2011-2013).

from 1-876 mg/lit (Krishan *al*., 2016). Results of present investigation also in conformities with the finding of previous authors in context of studied parameters. Besides this, results of present work are also supported by several workers, (Chapolikar, and Ubale 2010; Manjappa *et al*., 2011; Arya *al*., 2012; Nagarnaik and Patil, 2012; Kumar *et al.,* 2013; Chandne, 2014; Leelavathi *et al*., 2016; Prajapati and Rokde, 2016; Behailu *et al*., 2017; Soni and Singh, 2018).

According to WHO standard, potable water should be free of coliform bacteria (WHO, 2008) but the data from other study proves that the tube wells are commonly contaminated with faecal organisms (Luby *et al.*, 2008; Omezuruike *et al.*, 2008). It was found that out of the 454 samples, 49% (221/454) samples were contaminated with total coliform, ranges from 1.45±4.15 to 10780±33814 MPN/100ml and 14% (65/454) samples were contaminated with *E.coli* with concentrations ranges from 0.09±0.43 to 24.95±104.37 MPN/100ml and; 3% (13/454) of the samples were contaminated with *Salmonella species*, noticed as 0.06±0.41 MPN/100ml during analysing the status of groundwater contamination in Rural Area, Kelantan (Idrus *et al*., 2014). The total coliform contents of the samples ranged from zero to 16 MPN of coliform/ 100ml analysed during bacteriological assessment of selected borehole water samples in Ilorin metropolis (Agbabiaka and Sule, 2010). Whereas, the total coliform and faecal coliform were noticed in the range of 03-97 MPN/100 ml and 00-78 MPN/100 ml during microbiological analysis of groundwater of Khulais Province, Kingdom of Saudi Arabia (Saleem and Algamal, 2016).

Significant high coliform bacteria were noticed in borehole water, qualitatively correlate with levels of possible pollution in the immediate surroundings environment in some communities (Anima *et al.,* 2010). However, more or less similar patterns of total coliform (MPN/100ml) and faecal coliform (MPN/100ml) reported in present study are also in conformities with the findings of previous authors.

The present investigation can be concluded that there is a need of continuous monitoring of water quality and also proper environment management programs should be run to control drinking water pollution.

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Effect of time and frequency of static stretching on flexibility of rectus femoris muscle of human subjects

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ABSTRACT

The main aim of present study is to evaluate and compare the effect of frequencies and durations of static stretching on flexibility of rectus femoris muscle of human subjects. A sample of 50 volunteers having tightness of rectus femoris muscle was randomly allocated into five groups. Static stretching of rectus femoris muscle was performed in five different frequency and durations for 6 weeks. Group A (n=10) was subjected to 3 stretches for 30 seconds each once for 5 days in a week for 6 weeks. Group B (n=10) was given 3 stretches for 15 seconds each for 6 weeks. Group C (n=10) was given 1 stretch for 30 seconds. Group D (n=10) was given 1 stretch for 15 seconds. Group E (n=10) was given no stretch. The findings revealed that there was statistically significant increase in ROM(knee flexion)in first four groups A,B,C and D except Group E following the treatment with different parameters of time and frequency of static stretching during 6 weeks with mean difference = 6.60 (pre-interventionmean value & S.D of ROM=125.80±3.645& post-intervention mean=132.40±3.502), mean difference = 3.60 (pre-intervention mean=125.60±3.718 & post-intervention mean=129.20±3.718), mean difference=6.40 (pre-intervention mean=125.20 ± 4.104 & post-intervention mean=131.60±4.006), mean difference=3.60 (pre-intervention mean = 126.30±3.773 & post-intervention mean =129.90±3.479) & mean difference= 0.60 (pre-intervention mean = 125.10±3.784 & post-intervention mean = 125.70±3.561) respectively. Hence, the study exhibited stretching of rectus femoris muscle three or once for 30 seconds appears to be effective and showed significant improvement compared to 3 or 1 stretch for 15 seconds or no stretching. The results of Tukey test indicated that there is no significant difference between 1 stretch or 3 stretch for 30 second. The results of the present study showed that a one 30 –second duration is an effective amount of time to sustain a rectus femoris muscle stretch in order to increase ROM. No increase in flexibility occurred when the frequency of stretching was increased from one to three times per day.

KEY WORDS: FLEXIBILITY, RECTUS FEMORIS MUSCLE, STATIC STRETCHING, MUSCLE PERFORMANCE

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INTRODUCTION

Flexibility is defined as "the ability of a muscle to lengthen, allowing one joint (or more than one joint in a series) to move through a range of motion (ROM) (Bandy et al., 1994,1998) Roberts et al 1999) and a loss of muscle flexibility as " a decrease in the ability of the muscle to deform," resulting in decreased ROM about a joint. (Bandy et al., 1997, Russell et al., 2004). It is believed that stretching can prevent injury (Zachezewski et al.,1989 Hubley et al., 1984) enhance athletic performance (Anderson et al., 1991,. Beaulieu et al., 1981, Worrell 1994) and assist in rehabilitation following musculoskeletal injury (Agre et al.,1985). Stretching is traditionally used as part of warm up to improve flexibility (Shellock et al., 1985, McMillian et al 2006, Dalrymple et al 2010, Page 2012).

The literature document three methods of stretching exercises to increase flexibility: ballistic stretching, static stretching and PNF techniques. All three methods have been documented to increase ROM immediately after stretching. (Russell et al., 2004, Sady et al 1982). Proprioceptive neuromuscular facilitation involves the use of brief isometric contractions of the muscle to be stretched before statically stretching the muscle. This technique not only requires expertise to perform but also requires one-on–one intervention with another experienced individual. Static stretching is performed by placing muscles at their greatest possible length and holding that position for a period of time (Beaulieu et al., 1981, Anderson et al., 1991,Russell et al., 2004). Ballistic stretch uses bouncing or jerking movements imposed on the muscles to be stretched. The quick, jerking motion that occurs during the ballistic stretch can theoretically exceed the extensibility limits of the muscle in an uncontrolled manner and cause injury. The use of ballistic technique, therefore, has not been widely supported in the literature (Ferreira et al, 2007 & Torres et al, 2008).

Although documentation exists that static, ballistic, and PNF techniques will increase the flexibility of muscle, (Sady et al., 1982, Moore et al., 1980), but the most widely used method for increasing ROM because of the simplicity of execution and lower potential for tissue trauma. An ample volume of literature has focused on the effects of stretching on the flexibility of the hamstrings muscle. However,there is a scarcity of evidence of stretching on other muscle groups, such as the gastrocnemius, soleus, quadriceps and triceps surae, (Bandy et al., 1997, Russell et al., 2004). Rectus femoris is the only muscle in the quadriceps group that is involved in hip flexion, since it originates in the pelvis and not the femur. Isolated rectus femoris (RFM) tightness is associated with a variety of clinical disorders. Rectus femoris tightness may lead to alteration in swing phase, increased energy expenditure and pain during walking downstairs or when wearing high heels, (Csink et al., 1963, Lenart et al., 1974, Lateur et al., 1978 Richard et al., 1985).

Another important point of consideration in the present study is the effect of time and duration of stretching on flexibility of rectus femoris muscle. The duration and frequency of muscle stretching may interfere with the improvement of flexibility.While the literature is unanimous in its support for static stretching resulting in increased ROM, no consistency is apparent with regard to how long stretches (time) and how much stretches (frequency) should be held to obtain optimum benefits. Some researchers suggested that the 30 s duration of stretching with one or three times a day is effective for increasing muscle flexibility (Marques et al., 2009). However, other studies recommended that the duration of stretching in flexibility training programmers range from 5 to 60 seconds,yet justifications for these selections have largely been absent (Roberts et al., 1999)

The purpose of study was to determine the time and frequency of static stretching that most effectively increase flexibility of the rectus femoris muscles, as measured by active knee flexion test.The effects of five daily frequencies and durations of static stretching on rectus femoris muscle flexibility was compared 5 days per week for 6 weeks: (1) three 15 – second stretches (2) one 30 – second stretch (3) one 15- second stretch (4) a control (no stretching activity).The main objective of this work was to determine the optimal time and frequency of static stretching to increase flexibility of rectus femoris muscle, as measured by active knee flexion test.

MATERIALS AND METHODS

The present study was an experimental study (3 × 5 factorial designs), performed in the Research lab of Department of Physiotherapy at Shri Jagdishprasad Jhabarmal Tibrewala University, Rajasthan. The Institutional Ethical Committee approved the study, where prior to participation in the study, subjects were explained about the procedures that they would have to follow during the study. The patients' consent was taken on the consent form for participation in the study. All the subjects were the residents of Rajasthan, who volunteered for the study and were randomly allocated into five groups. The size of the sample was 50 subjects with 10 samples each in group A,B,C,D and E. Subjects of either genderaged20 to 30 years having unilateral (right side) tightness of rectus femoris muscle with no history ofback pain, urinary tract infection, soft tissue injury, fracture of lower limb, inflammatory joint & arthritic condition of lower limb with acute or chronic rectus femoris injury were

recruited. The subjects must exhibit positive knee flexion test with positive knee flexion ROM between 120-130 ± 2 degree. In addition, subjects should not have involved in any lower extremity stretching exercises during 6 week study. The list of variables include time and frequency of static stretching, flexibility of rectus femoris. Universal goniometer with a double – armed, full – circle protractor made of metal was used for all measurements. Rectus femoris muscle flexibility using active knee flexion test of each subject was measured prior to assignment to groups and the reading for ROM were taken once after 6 weeks of intervention. The subject was placed prone, with both feet off the end of the examination table. The knees were extended and the hips positioned in 0 degrees of flexion, extension, abduction, adduction, and rotation.

The hip was stabilized to maintain the neutral position. The hip was not allowed to flex, a cloth strap was then placed securely across the buttocks to stabilize the hips in extension, when the strap was tightened the subject was instructed to adjust his body to a comfortable prone position, because tightening the strap caused the trunk and hips to rotate. With the hips stabilized in extension and the ankle relaxed in plantarflexion over the edge of the table, the subject was told to slowly bend his knee to the point of "initial resistance". When the end point was reached, the degree reading of knee flexion using universal goniometer was observed and recorded.

Following the initial measurements, subjects were randomly assigned to one of five groups. Subjects assigned to group 1 (6 men, 4 women mean age = 24.70 years,SD= 2.60) did three 30 seconds static stretches (10 seconds between stretches) of the rectus femoris muscle. Group 2 (6 men,4 women mean age = 24.30 years, SD = 3.12) did three 15 second static stretches, with a 10 second rest between stretches. Group 3 (4 men, 6 women mean age = 24.40 years, SD= 2.79) did one static stretch for 30 seconds. Group 4 (4 men, 6 women mean age =24.40 years, SD = 3.27) did one static stretch for 15 seconds. The fifth group (6 men, 4 women mean age=24.50 years, SD= 2.87) served as a control group and did no stretching activities.

Subjects in group 1 through 4 stretched 5 days a week for 6 weeks.To stretch the rectus femoris muscle, each subject stood upright with one hand against a wall for balance under supervision of the therapist. The knee was flexed and the hip extended, the lumbar spine and pelvis were aligned in a neutral position. The pelvis did not tilt anteriorly nor the low back hyperextend; the hip was not abducted or remained flexed. When the subject did self- stretching ,the trunk was erect,not slumped. the subject then flexed the dominant leg to a knee joint angle as possible. and the stop watch was used to calculate the accurate timing (30,15 and 10 sec).

After that all subjects were retested after 6 weeks using same procedures described for the pretest.

RESULTS AND DISCUSSION

The results have been explained under the following sub-headings: 1) Effect of static stretching on flexibility of rectus femoris muscle in term of ROM (knee flexion) with different parameters of time and frequency during 6 weeks:

The primary objective of the current study was to investigate the effect of time and frequency with different parameters of static stretching on the flexibility of rectus femoris muscle. Therefore 4 different parameters of static stretching were administered for the duration of six weeks, namely 3 stretches for 30 seconds, 3 stretches for 15 seconds, 1 stretch for 30 seconds and 1 stretch for 15 seconds once for 5 days in a week. The findings revealed that except the control group, all the four groups showed statistically significant improvement in muscle flexibility.

Stretching the rectus femoris muscle at 3 or 1 stretch for 30 seconds appeared to be effective and showed significant improvement compared to 3 or 1 stretch for 15 seconds or no stretching (control). As indicated by the Tukey test,there is no significant difference between 1 stretch or 3 stretch for 30 seconds. Therefore, 1 stretch for 30 second is a sufficient stretch to increase the flexibility of rectus femoris muscle. The results of this study are consistent with the results of another study conducted by Bandy et al., 1994 comparing the effect of time and frequency with different parameters of static stretching on hamstring muscles,in which stretching for 30 second one or three times per day for 5 days per week for 6 weeks was more effective for increasing muscle flexibility than no stretching. There was no difference between stretching one or three times per day using either a 30 – or 60 – second duration of stretching. The use of longer duration and more frequent daily stretching, therefore must be questioned.

Gains in flexibility involve biomechanical,neurological and molecular mechanisms that determine myofibrillogenesis as a long-term result.The gains in flexibility can also be associated with increased tolerance to pain and increased viscous elastic properties of the muscle-tendon units. Several studies have reported flexibility and ROM after stretching programs and the present results support these findings.The results of this study,in conjunction with previous research ,showed that the increased the duration and frequency beyond one 30 –second stretch performed one time per day did not increase flexibility and it indicated that 30 seconds is an effective length of time to sustain a rectus femoris muscle stretch in

Table 1. Pre and Post intervention comparison among five groups; Group A, B, C, D and E

Groups	A (n=10)	B (n=10)	C (n=10)	D (n=10)	E (n=10)
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
Pre- values	125.80±3.645	125.60±3.718	125.20±4.104	126.30±3.773	125.10±3.784
Post-values	132.40±3.502	129.20±3.718	131.60±4.006	129.90±3.479	125.70±3.561
Mean difference	6.60	3.60	6.40	3.60	0.60

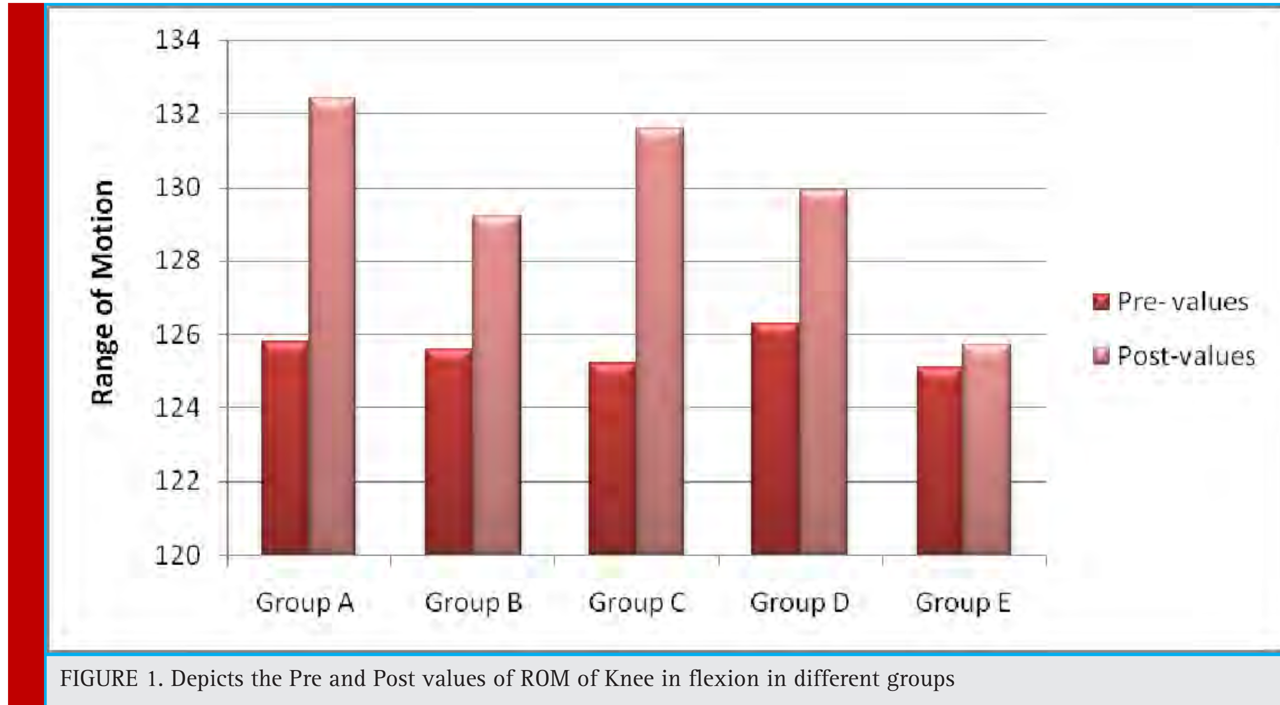

FIGURE 1. Depicts the Pre and Post values of ROM of Knee in flexion in different groups

Table 2. ANOVA analysis of ROM (knee flexion) for all five groups.

Variable	Sources of Variation	SS	Df	MS	F-ratio	Significance
Range of motion (ROM)	Between	242.72	4	60.68	195.0429	Significant
	Within	14	45	0.311111		

order to increase ROM.This observation is clinically relevant,suggesting that one 30 –second stretch performed five times a week is sufficient to improve flexibility compared to stretching at a higher frequency.

This study contrasts with the findings of Bandy and Irion (1998) who found stretching durations of 30 and 60 seconds to be more effective than 15 seconds. However, in the study of Bandy and Irionthere is no indication whether total amount of time spent in a stretch position during the entire training programme was controlled. This factor, if not controlled, could explain the differences observed subjects in the longer duration treatment groups would be exposed to more time in a stretched position and therefore likely to show greater improvements. The present study did control the total amount of time spent in a stretched position during the entire training programme.a stretching duration of 15 seconds resulted in significantly smaller improvements in active ROM than a duration of 30 seconds, suggesting that 15 seconds may not be long enough to elicit a Golgi tendon organ response.

Table 3. Pair wise comparison of group means in all five groups in term of ROM (knee flexion) during 6 weeks

	A		
B	Significant	B	
C	Non-Significant	Significant	C

Another study of Bandy et al showed that 30-second static stretch performed one time per day over a 6 week period resulted in more than twice the gains in hamstring flexibility than performing dynamic range of motion at the same frequency and duration. Only few of the studies have been done on the flexibility of rectus femoris. Therefore, such studies contribute little toward understanding the mechanisms behind the acquired increase in ROM after stretching.

CONCLUSION

Our study demonstrated that although stretching for 30 seconds one or three times per day for 5 days per week for 6 weeks was more effective for increasing muscle flexibility (as determined by increased knee flexion ROM) than no stretching or 15 sec stretch, there was no difference between one or three times per day using 30 – second duration of stretching. Therefore, 30 – second duration is an effective amount of time to sustain a rectus femoris muscle stretch in order to increase ROM. The results from this study will be helpful for individuals who desire to increase their flexibility in an attempt to decrease injury and enhance performance, as well as for those clinicians who incorporate static stretching activities as part of their rehabilitation programs.

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Real time deployments of sensors and sensing patients vital parameters using secure autonomous WSN for medical applications

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ABSTRACT

Wireless Medical Sensor Networks (WMSNs) have emerged as the most reliable technology for implementing a pervasive paradigm, facilitating doctor to patient efficiency and improving the quality of life. Sensor devices have invaded the medical domain over a recent past with wide range of capability. WSN for healthcare enable remote patient monitoring, timely exchange of health information, reminders and support thereby extending the reach of healthcare assistance anywhere, anytime. In this paper, we analyze the performance of a scalable WSN infrastructure with respect to medical applications and presents their response in scenarios which are simulated to mimic real-time behavior. All simulations have been done in MATLAB. Proposed design of an autonomic WMSN in such a way that it meets the requirements of various applications like sensed quantities, body sensor nodes autonomy, energy efficiency and reliable transmissions. The focus of the paper is on the overall network efficiency since low energy consumption, increased throughput and reliable transmission are the prerequisites for providing robust, reliable and long-lasting unhindered operations in healthcare. From the results, it shows that our autonomic WSN tries to maintain optimal amount of power for each node and also ensures that appropriate data communication occurs for all the nodes in the network.

KEY WORDS: WIRELESS MEDICAL SENSOR NETWORKS (WMSNS), WIRELESS SENSOR NETWORK (WSN), HEALTHCARE MONITORING, AUTONOMIC SYSTEM. MESH NETWORK

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INTRODUCTION

Wireless medical sensor networks (WSNs) are among the most promising technologies that enable enhanced sensing capability, powerful data processing and increased communication ability from human body, within various environments and in different context. The technology offers a unique set of capabilities that produces an exciting but complex design space which is often difficult to negotiate in an application context. Deploying sensing physical environments has its own set of challenges, and can result into the system failure, thus resulting in problems that can be difficult to discover or reproduce in simulation. Sustained efforts in the area of wireless networked sensing over the last few decades have resulted in a large number of theoretical developments, substantial practical achievements. In order to bridge the gap between (on the one hand) a very large scale, randomly deployed, autonomous networks and (on the other) the actual performance of fielded systems, we need to consider deployment as an essential component in the process of developing sensor networks: a process that serve specific applications and end-user needs. Incorporating deployment into the design process reveals an entirely new and different set of considerations and requirements whose solutions require innovative thinking, strong involvement from end-user communities and multidisciplinary teams.

WMSN face particular challenges. They are deployed in hostile environments and are expected to operate efficiently within very confined technological limitations, not least of all in respect to power requirements. Maintaining the operational lifespan of the WMSN is a fundamental objective as we cannot afford to lose the medical data being transmitted in the network. While the use of intelligent techniques offer one approach, this paper will advocate the adoption autonomic principles, augmented with intelligent techniques, as the primary means by which this objective is met. The paper is structured as follows: Section I provides a brief description of WMSNs. In Section II, the notion of an autonomic WMSN is examined, with particular emphasis on the critical issues of intelligent power management, and intelligent coverage and reliable packet transmissions via message switching respectively. Section III describes the overview of the technology used. Section IV, V, VI provides the detailed motivation, architectural design and implementation of our idea respectively.

The paper specifically addresses issues of generic importance for WMSN system designers:

(i) Autonomic Behavior, (ii) Energy Efficiency, (iii) Communication Availability and Quality, (iv) Timely Data Transmission of the network designed. The main focus is the deployment and deployment evaluation.

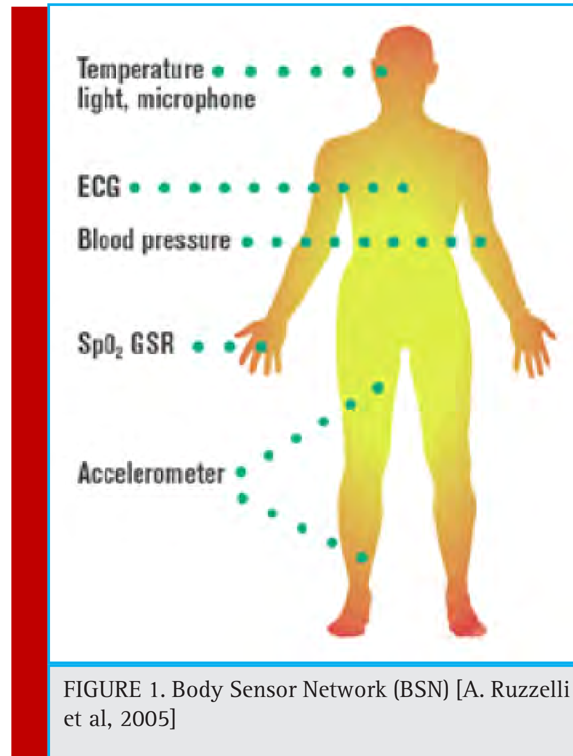

FIGURE 1. Body Sensor Network (BSN) [A. Ruzzelli et al, 2005]

In this work, MATLAB has been used to simulate and analyze the performance of a scalable WMSN infrastructure with respect to medical applications such as pulse rate, body pressure, temperature etc. and find out how such systems respond in scenarios which are simulated to mimic real-time behavior.

AUTONOMIC WSN FOR MEDICAL APPLICATIONS

With the dramatic increase in computing devices, their increased computing capacity and complexity combined with popularity of internet, there has been a phenomenal growth in heterogeneous networks and network applications. Due to this increasing complexity, network management issues and communication protocols have reached a level beyond human ability to manage computer systems. At the highest level, the solution is to have computers manage themselves. This is achieved by providing pre knowledge to a computing element about its operation that it is capable of making informed decisions leading to its automation, self- protection and self-management.

Based on the inspiration from the autonomic functioning of human central nervous system, where autonomic controls use motor neurons to send indirect messages to organs at a sub conscious level, an initiative was created by IBM towards Autonomic Computing for relieving humans from the burden of managing computer systems which is enormously growing to the extent of unmanageability.

TECHNOLOGY OVERVIEW: DEPLOYMENTS AND SENSING OF MEDICAL SENSOR NODES

WSNs often lack robustness, are unreliable, have many elements which interoperate in complex manners, and are subject to much environmental variability. This means that it would be extremely difficult for a person to effectively administer a WSN (if access is even possible); therefore, system administrators would benefit greatly by the implementation of autonomic principle to a system (Kephart et al., 2003 and Ruzzelli et al., 2005)

To clarify the contribution that autonomic computing can bring to WMSNs, we will provide a series of scenarios in which a common problem in WMSN operation can be tackled using autonomic principles.

Self Configuration

Firstly, there is an issue of deployment. It is often assumed that the nodes forming a network cannot be perfectly positioned. Hence, a pre-programmed configuration for the network will not work. Self-configuring nodes can set up network connections, proceed to establish sensing and communication schedules and evaluate if there are any gaps in the WSN, either from a networking or sensing viewpoint. This can be well understood by studying a protocol (Ruzzelli *et al*, 2005) for automatically building a network out of randomly distributed nodes.

Self Protection

Secondly, there is the self-protection attribute. Sensor nodes are usually exposed to harsher environmental conditions and are thus, subjected to energy depletion and incidental damage which can lead to gradual degradation of the network as a whole. Network paths break and gaps appear in the sensing coverage of an area. A WSN needs to adapt to the changes in its topology constantly throughout its lifetime.

Therefore, WSN needs to adapt to the changes, recover from losses and be self- protected. This is achieved by renegotiation of network routes, voltage level monitoring with the sensor nodes, control over each node by base station and upon failure activating redundant nodes to replace the damaged ones.

Self Healing

Thirdly, self-healing is the ability to detect and eliminate the damage caused to the network transmissions due to the addition of some unwanted elements. (e.g., sensor transmission contaminated with some noise).

Self Optimization

Lastly, self-optimization is an important trait for WSN protocols so that maximum energy efficiency is gained from the available energy as the energy at each sensor node is limited. Given an application that uses the network, energy savings can be achieved by reducing

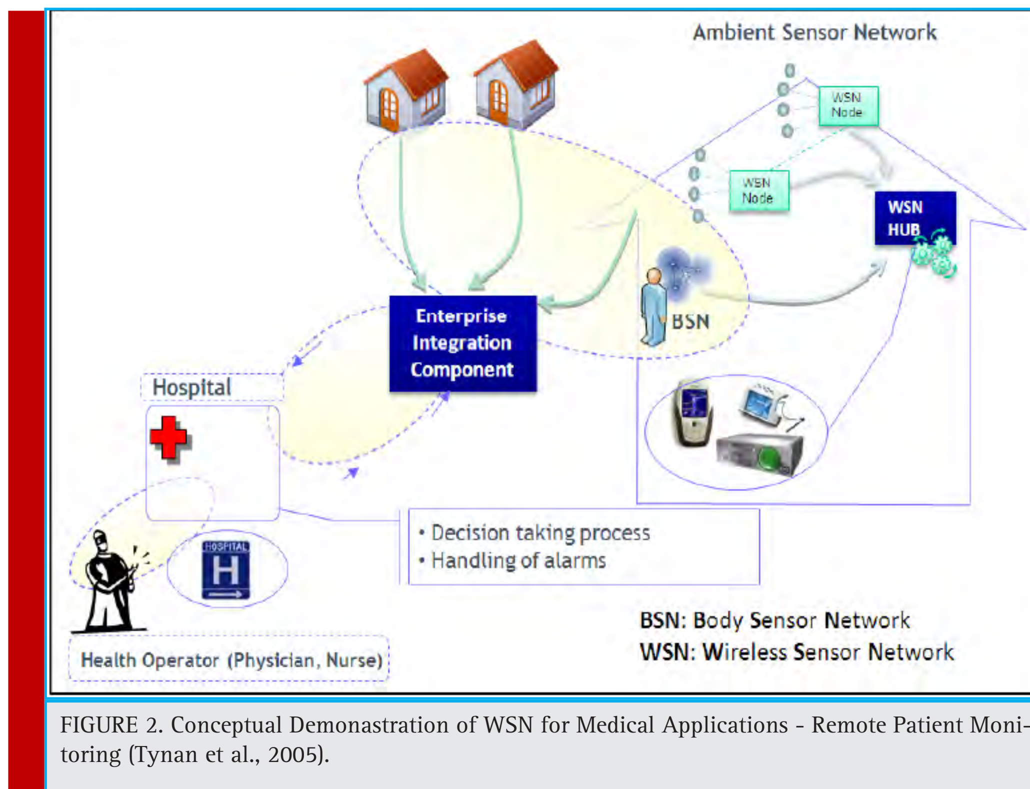

FIGURE 2. Conceptual Demonastration of WSN for Medical Applications - Remote Patient Monitoring (Tynan et al., 2005).

the overall performance of the network and because redundant nodes can be put into a low-power sleep mode, ready to be reactivated when the need arises. This can prove to be the best effort in achieving maximum energy efficiency. In the following subsections, we will expand on the details of the most important aspects of a WSN, namely network integrity, sensing quality and power management, and how autonomic principles aid in their optimization.

Intelligent Power Management:

Due to the deployment of WSN in a potential hazardous environment, it is of paramount importance that the network operates for as long as possible without requiring routine and frequent maintenance tasks. The most common of these tasks is battery replacement. Thus, it is vital that the nodes of the network manage their power consumption in an intelligent manner to deliver the longevity required by the network. A node's lifetime will be proportional to the amount of time it is active. Therefore limiting the nodes activity on a network wide basis will increase the lifespan of the entire network and this has proved to be one of the most effective power management techniques for WSNs [F. Ye *et al*, 2003]. An inactive node is termed as *hibernating,* a temporary state in which negligible or no power is consumed by it. Hibernating nodes are unable to report their sensed data and as such leave a blind spot in the network, where no active sensor is monitoring. The hibernation of a node must be performed in context to a network wide quality of service metric that must be maintained by the active nodes. For power management, this is typically coverage. Each sensor is associated with a sensing radius within which it can sense and outside which it is dormant. Some techniques use an inverse distance relationship between the sensor and its ability to sense but there is still a limit to its sensing capability. An area is deemed covered if the union of all the active sensors sensing discs includes every point within the sensed area. This is the constraint under which nodes may be hibernated, thus managing the networks power consumption intelligently while maintaining a quality of service with regard to its surveillance density. Algorithms based on this principle include CCP [H. Zhang *et al*, 2005] and OGDC [F. Ye *et al*, 2003]. These techniques operate on an optimization of the problem, ensuring that the intersection of two sensing discs is covered by another sensor and that every intersection point of the sensors discs within the boundary area are also covered by another sensor. Using this property the algorithms manage the nodes activity and limit the power consumption by the network. Another technique based on the standard coverage maintenance is interpolation, and more specifically interpolation error [R.Tynan *et al*, 2005]. For a given sensor network meant for sensing temperature for example, there will be a temperature distribution function across the area of the network. Sensors of the network are responsible for sampling this function at discrete locations within the area and report their readings to a base station. Interpolation is a mathematical technique for approximation of function values between known values of that function. The known points of the temperature function are at the locations of the sensors within the context of sensor networks. Therefore, interpolation could be used to approximate the temperature between the sensors. A set of sensors used to approximate another sensor's temperature reading and then comparing it to the actual reading at the sensor's location is the interpolation error. Hibernating sensors whose interpolation error is less than a particular defined application threshold, then the node is hibernated, thus conserving further amounts of energy. This interpolation technique is the first coverage technique that includes neighboring node locations as well as neighboring node readings and can deliver greater savings.

Intelligent Routing:

Forwarding of packets from a source to a destination is the fundamental feature of multi-hop networks. In such networks, a node should be capable of identifying the best node to pass on the packet so that it reaches the destination in time. The easiest way to do it would be to flood the entire network; however, this is an unfeasible approach due to the energy consumption and packet overhead that the flooding would cause. An efficient routing poses great challenges such as which node to forward the packet among all neighbouring nodes and the trade-off between lightweight (but with high delay) reactive on-demand type routing and low latency (but computational heavy) of proactive type routing. Proactive protocols try to keep an accurate snapshot of the networks working status and maintain full knowledge of the system; hence they are suitable for high computational capability devices but the low processing capability of sensors prevents them possessing a full proactive routing protocol. On the contrary, on-demand routing protocols exempt from maintain a routing table all the time but only build it when a packet needs to be relayed to a certain destination. This is not an easy task, especially when the destination is unknown, and the situation is exacerbated when sensor node energy constraints do not permit continuous network flooding of packets. A secondary issue concerns global node addressing, which causes to prevent functioning of nodes in two different parts of the networks from having the same ID; an undesirable situation which can lead to incorrect routing for certain destinations. Apparently, this is not a trivial problem, particularly when dealing with large scale sensor networks that may comprise thousands of units. The

literature survey reveals a profusion of routing protocols, all tailored to wireless sensor networks. However, before delving into the relevant approach proposed, it is important to understand the necessary perquisites for a routing algorithm to be apt for sensor networks:

1. Self-organization, as the expended nodes might or might not be substituted with new ones; hence it is important for the network to be prepared in advance for sudden changes of devices and possibly their location.
2. Flexibility, external factors such as temporary or permanent obstacles, might cause the network topology to change continuously due to which neighboring nodes may be disconnected. This can result in the need for the identification of a new routing path.
3. Scalability, a sensor network consists of a certain number of nodes that may span from single figures to tens of thousands of devices.
4. Lightweight, due to limited processing capability single devices may be prevented from having a high computational load.
5. Energy-efficiency, nodes run on batteries and need may arise to scavenge energy from the environment. Furthermore, nodes might be deployed in remote areas where it is impractical to recharge them.
6. Loop-free, the routing algorithm should ensure that the packets are not routed endlessly around the network without finding the correct destination.
7. Reliability, the protocol should guarantee a high percentage of correctly routed packets to the destination.
8. Tolerable latency, some application need to receive the packets requested within a certain deadline. Thus, it would be preferable to have a protocol that can autonomously trade off energy savings for packet delay as per the application needs.

All the above characteristics imply the need for an Intelligent Routing protocol for autonomic wireless sensor networks (Ruzzelli et al., 2005).

Intelligent Coverage:

One of the most important aspects of a sensor network is to have satisfactory coverage i.e. the ability to provide sensory data of sufficient quality to the application which is using it, At the highest level, what concerns the most is that the sampling frequency, both in spatial and temporal terms, is high enough so that the phenomena of interest can be observed in sufficient detail. However, considering WSNs, we must also be aware of the energy cost of any actions. As suggested earlier, it is necessary to balance the level of detail the network is providing to the client against the rate at which energy is being consumed while gathering the data. Clearly, it is preferable to tune the network automatically, rather than doing it manually. Autonomic computing helps in intelligent reasoning about the coverage in the network. Various methods can be used to change the quality of the sensory data the network produces. The simplest among them is to reduce the rate at which the sensors sample the environment. This increases the time period the sensor nodes can spend in a low-power sleep state, and is relatively easy to coordinate. A more complex mechanism is to identify nodes that are not required due to a high density of nodes in a particular area [H. Zhang et al, 2005]. This needs coordination between the nodes on a local level to decide which ones should sleep and which ones should enter an active state. If the range of the sensors is variable, then varying this parameter can be put to use is one more way to fine tune the power/sensing relationship [D. Marsh et al, 2005]. When flooded with pre-calculated knowledge of the relationship between deployment densities, coverage levels and sensor ranges, a node can alter these variables while avoiding combinations that would have an adverse effect on the network as a whole. Finally, mobile nodes have the capability to reposition themselves as conditions dictate [K. H. Low et al, 2006]. In a mixed network of fixed and mobile sensors, the preservation of coverage in areas where too many static sensors have become depleted or damaged (often considered a failure condition for WSNs) is facilitated by this. In mobile networks, nodes effectively occupy two or more spatial locations once they switch positions faster than the requisite temporal sampling rate, thus restricting down on the number of nodes needed. A special case occurs when an inherently static node with independent decision making is attached to a mobile object, for instance a vehicle or person. In this instance coverage becomes a probabilistic problem, since no actions on the WSN's part can influence where the nodes will end up. In all types of coverage that a WSN requires, there exist trade-offs. It is often possible to reduce communication rates for an increase in processing time. When mobile nodes are equipped with an appropriate sensing modality, sensing can be utilized to observe other nodes, rather than using the radio to exchange positions (Low et al., 2006). Thus, depending on the relative costs of sensing, processing and radio communication, a sensor network can dynamically choose to favour one method over the other if it leads to significant energy savings. By intelligently choosing the optimal alternative, an autonomic sensor network can achieve energy savings to a level beyond what could be expected from a standard WSN.

MOTIVATION AND PROPOSED SYSTEM MODEL

Modern wireless sensor technologies enable powerful data processing, enhanced sensing capability, and increased communication ability from human body, in different context within various environments. With increased prevalence of chronic diseases all over the world, there is constant pressure on healthcare system to find ways to deliver reliable healthcare solutions that can provide same service at affordable cost and the services that do not need intensive enrolment of medical staff. In critical emergency response scenarios, designing a medical sensor network that can deliver suitable functionality (e.g. energy efficiency, throughput, sensor data transmission rate) to meet the evolving patient, provider, and workflow needs is a critical challenge. The use of wireless technologies in medical environment is bringing major advancement to the existing healthcare services. However, these have several key research challenges such as various types of network communication infrastructure, fault-tolerance, data integrity, low-power consumption, transmission delay, node failure, etc. Reliability is one of the most important factors in a successful healthcare system. To ensure this factor, system designers have to care about adaptation of nodes when its location, connection and link quality is changed. Different network communications infrastructure should be used in appropriate situation. For example, with high-risk patients, higher QoS services should be used. The integrity of distributed data system and fault-tolerance should be given a proper consideration also. Every device operates differently at different times, especially sensor devices. A node in a network can be fail at anytime for number of reason including battery exhaustion, human-related issues or natural issues. Ensuring a seamless service during life time of the network could be a big challenge. How to manage the transmission delay of various types of communications in the network is an undoubted challenge. With the system using WMSN, data must undergo reduced hop counts before it reaches the sink. In addition, these hops are sometime located in very critical conditions, such as areas bearing interference of radio waves or magnetic field. As a result, various transmission delays can occur and thus, require extra effort of system designer to synchronize the whole network.

While deploying a WMSN all sensor nodes are communicating to the central server i.e. hub through a wireless protocol. If the transmitted packets containing patient data are not routed properly due to routing congestion, there is likelihood that the packets containing patient's vital information will reach late or not reach the desired destination at all. This leads to packet delay which is strictly undesirable in WMSN as diagnosis delayed is as good as diagnosis not done. Also the problem of packet delay is due to the coverage area as medical sensor networks consists of millions and billions of sensor nodes deployed within a particular range. Suppose the range is x metres and the nodes in this particular range become so dense that communication is effected to an extent that leads to packet delay. To counter attack this situation our priority is to reduce the effective area of the network by finding a common balance between the number of nodes in a given region and the number of packets they will transmit. Further, the sensor nodes deployed in the network are continuously communicating altogether at the same time. Thus, there becomes a possibility that some of the nodes become vulnerable and might get hacked.

We are introducing a dynamic medical sensor network architectural paradigm, where any of the routing protocol and security algorithms have the ability to tune themselves to suit the usage scenario. The sensor network can be statically configured prior to deployment and dynamically reconfigured during operation.

VERACIOUS WIRELESS MEDICAL SENSOR ARCHITECTONICS –VWMSA

As a solution for listed problems we have proposed and created VWMSA – Veracious Wireless Medical Sensor Architectonics The aim of VWMSA is to serve as a very simple but robust wireless sensor architecture that can help in designing a reliable, scalable and fault tolerant topology meant for WSN. An efficient architecture should be capable of offering:

- High throughput
- Small overhead
- Low or manageable latency
- Optimal power consumption
- Excellent ultra-low power performance in continuous reliable transmission of data, and large data traffic.

In order to accomplish all the above goals we create a WSN topology – VWMSA which is intended to provide energy efficiency communication, both for reliable transmission of data, and for data streaming. It incorporates *autonomic behavior* which is very much desirable in modern WSN also a method of packet switching known as *message switching*. Thus, it contains proprietary communication solution methods which are ideal for medical applications as we can't afford to lose any data being transferred in the network.

VWMSA shows an autonomic behavior where the central server can place the nodes into active and sleep mode but once the nodes are active, they can take their own decisions in real time. Also the nodes can com-

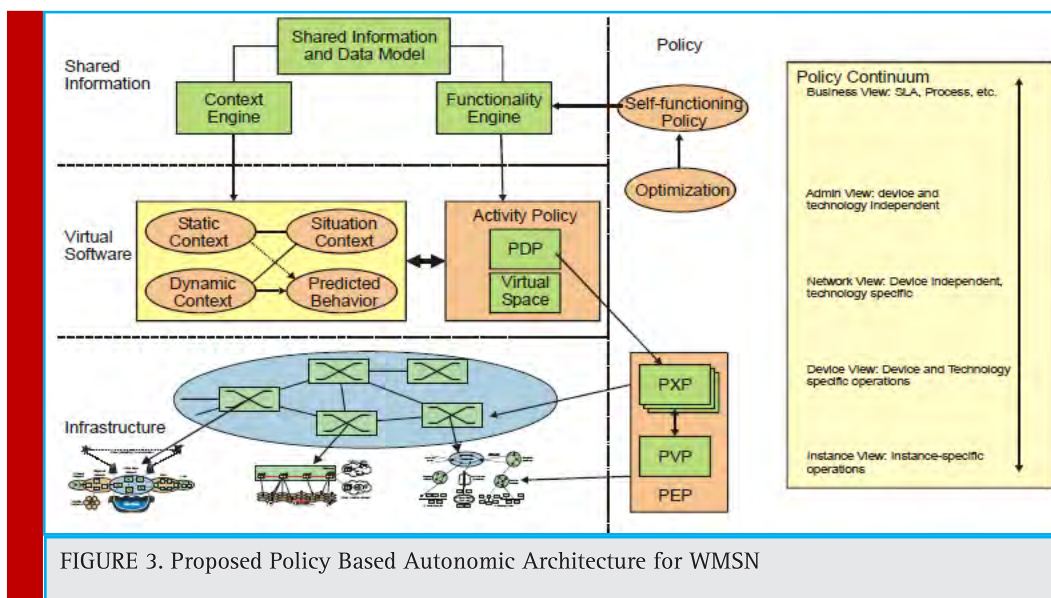

FIGURE 3. Proposed Policy Based Autonomic Architecture for WMSN

municate with each other in real time through a packet switching technique *called message switching* where the packets are routed from the source node to the destination node, in their entirety, one hop at a time.

In message switching [Cohler *et al*, 1967], the source and destination nodes are not involved in direct communications. Instead, the intermediary node (central server) is responsible for transferring the data from one node to the other by the process of activation/deactivation of the source node. It is only after the source node is set up into transmission mode i.e. when it receives the clearance or acknowledgement from the destination node, packets are transmitted in just one go preventing malicious packet to enter the network. As shown in Figure 4 data packet P1 is transmitted from A – C and data packet P2 is transmitted from D – B. Data P1 follows the route A →I→ II→III→C and P2 follows the route D→IV→II→B depending upon the availability of the free path at that particular moment.

IMPLEMENTATION AND PERFORMANCE ANALYSIS OF WMSN

The purpose of this prototype is to assess the performance of a Wireless Sensor Network primarily targeted for medical applications. Our primary concern is to verify that all the nodes are always able to communicate their data to the main server and that network performance is fairly uniform over the entire duration of the operation (defined by simulation time). Our approach is to create coherent wireless sensor network architecture with arbitrary number of nodes assumed to be scattered in a 2-dimensional space. The next step is to define a proper topology that can ensure that the data is always safely transmitted and shared within the network. For this we create an adaptive segmentation of regions each having a finite number of nodes i.e. continuous changes with each time stamp to avoid security vulnerabilities. During this region wise segmentation, all the nodes will be used

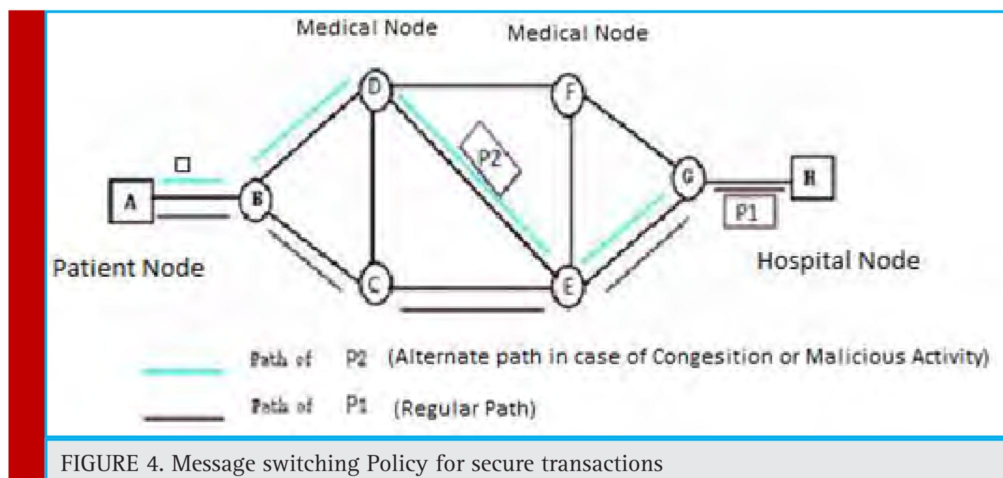

FIGURE 4. Message switching Policy for secure transactions

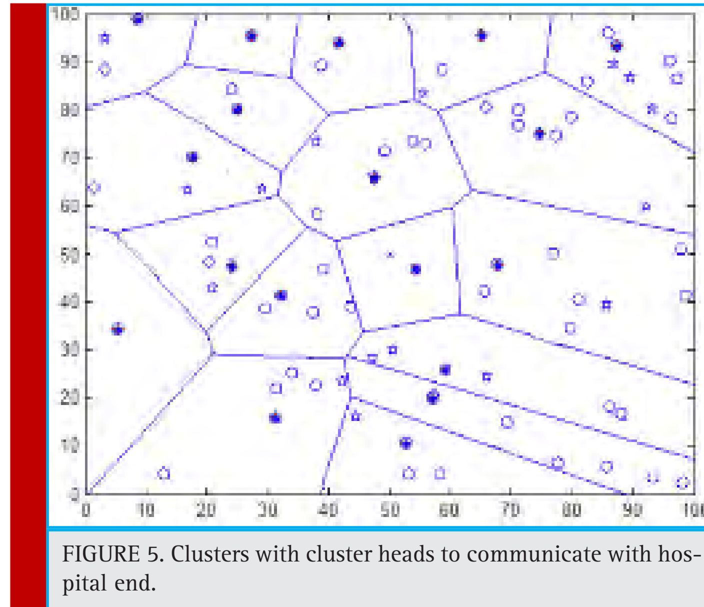

FIGURE 5. Clusters with cluster heads to communicate with hospital end.

to communicate with each other through packet transmission. It is assumed that only one packet is transmitted every time stamp. Fig.5 shows the clustering of the various sensor nodes representing various hospital data. The region wise segmentation is performed based on the real-time data being transmitted and how important the data is. These regions are guaranteed to have reliable operation since network traffic is optimal. We intercon-

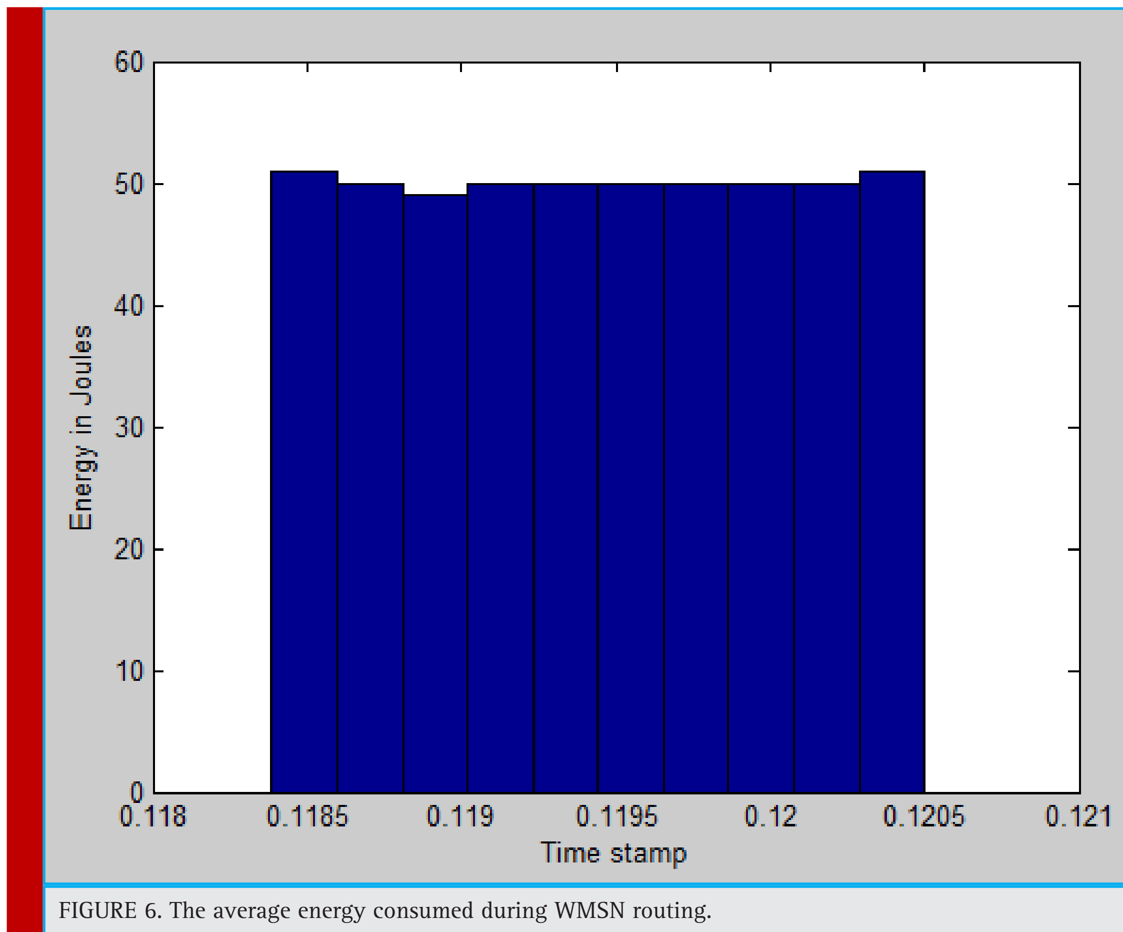

FIGURE 6. The average energy consumed during WMSN routing.

nect these regions to form a global network thereby assuring optimal performance of the entire WMSN by overall reducing the effective area of the network.

Once the operation of the WMSN is simulated (using event driven simulation) we collect several important parametric data and use it as benchmarks for assessment of the WMSN model created and check its reliability in terms of energy efficiency, clustering density and throughput analysis. The efficiency of the proposed approach is based on the simulation studies that have been performed using MATLAB®.

Energy Efficiency

The average power consumption of the sensor nodes and the network lifetime is related to the work-idle-work intervals. Evidently, the longer the intervals lower the power consumption. The adaptive working mode can be applied in most of scenarios in WMSN. For increasing the network lifetime, nodes are configured to "Sleep" or power saving mode in which the sensor nodes could turn off most of the modules, greatly reducing quiescent power consumption under idle state. In contrast, data-intensive nodes are configured to the "Continuous" or "Standby" power saving mode in which the sensor nodes work continuously, for data collecting and transmission. When the communication process is done we have to find the measure of the network performance where the communication is done satisfactorily and all the nodes are performing well. This depends on the amount of energy being consumed in the network and it should be uniform across all the nodes for the communication to take place reliably.

Cluster Density

Hierarchical based clustering seems to be ideal for WMSNs. It ensures energy efficient routing by forming local clusters and transmitting the information to the gateway nodes based on the events incurred in an adjacent area followed by data aggregation by means of the gateway nodes. The clustering and re-clustering designs are simple and reliable [Sung-Hwa Hong *et al*, 2013].

Cluster head selection is performed in a greedy manner via the local exchange of node energy states. Each cluster head determines when to abandon its role and become a cluster member, depending only on its own energy state.

These local interactions and local decisions regarding clustering and re-clustering increase scalability and reduce control overhead at the cost of reduced optimality. The clustered structure is not for the routing purposes.

Routing information is managed independently from cluster structure. A cluster member transmits packets only to its local cluster head, but a local cluster head can transmit packets to any nodes that can route the packets to the central server or the hub node which is where the

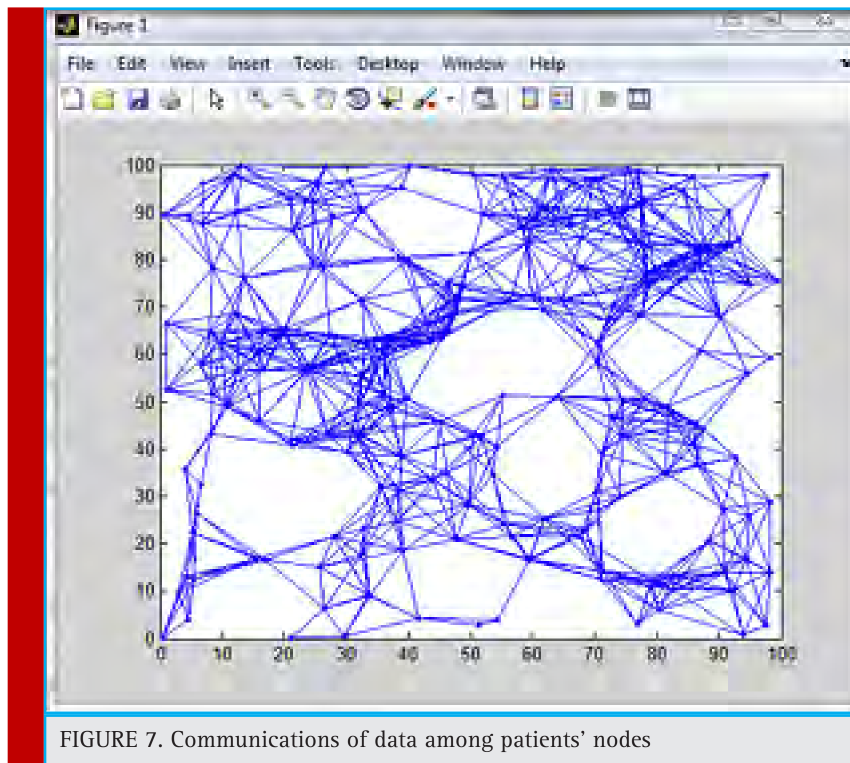

FIGURE 7. Communications of data among patients' nodes

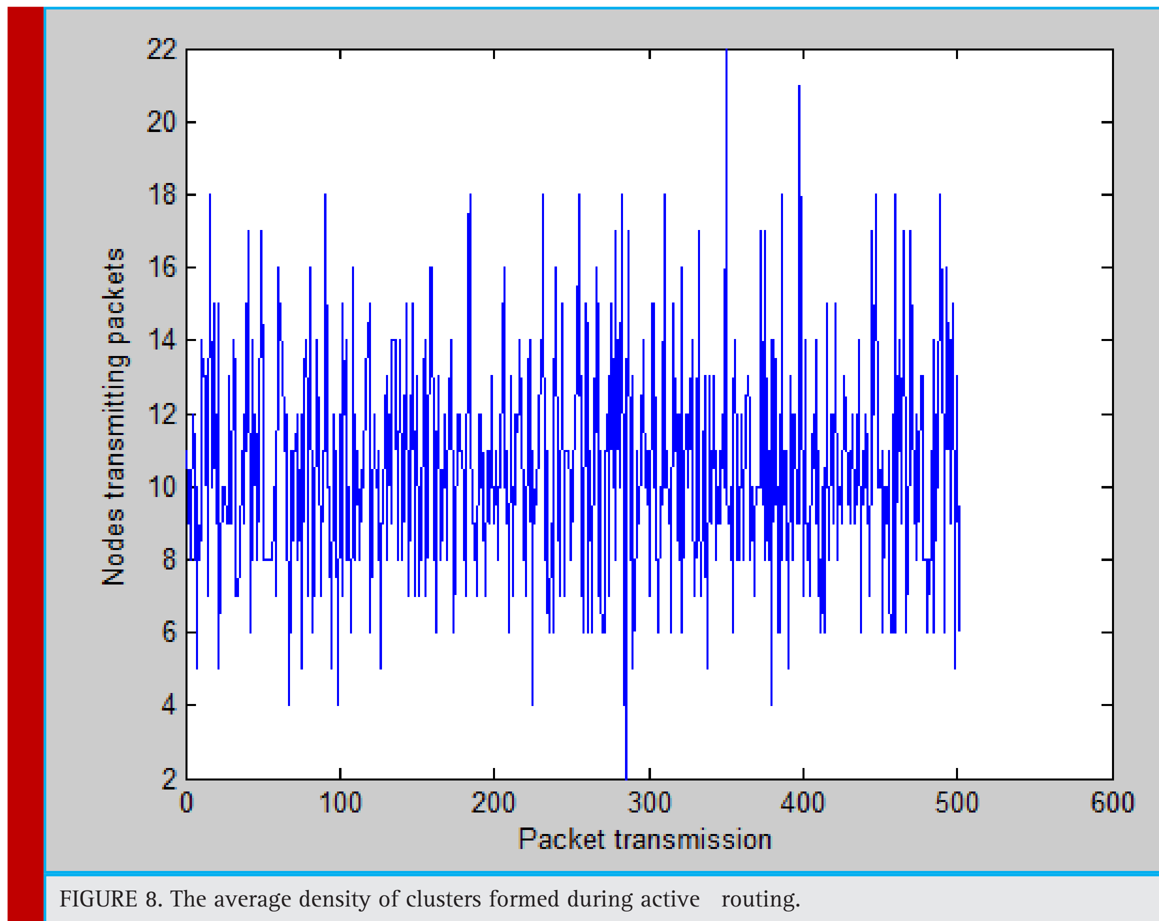

FIGURE 8. The average density of clusters formed during active routing.

doctor has an access to the patient's data. Fig.7 shows the average density of clusters being formed while active routing is being performed.

This has similarity to a random variable which is not surprising as multiple routes can be used in the WMSN for delivery of the packets.

Throughput

The percentage of total packets received successfully, is known as throughput of the network or packet delivery ratio. It is expressed as:

In Fig.9, almost all nodes show uniform packet transmissions since, they send relatively greater number of packets in the network. The plot shows the average throughput of the sensor nodes in a given region specifying how the data packets are uniformly distributed with respect to the time.

CONCLUSIONS AND FUTURE WORK

Wireless sensor networks for healthcare certainly have developed to a stage where their usefulness in healthcare application is undoubted, but the technology is still at an early stage of development. Problems that need to be solved in order to facilitate the use of the sensor networks in medical environment are lack of standardization and therefore low interoperability. In this paper, a relaying energy-efficient heterogeneous WMSN architecture for patient monitoring is proposed. This architecture compatibly incorporates various routing protocols and security algorithms that define the minimum energy parameters for the sensors to avoid damage to the human lives. Main focus lies on the autonomic architectural design of WSN for medical applications. The results are satisfactory and clearly show that in terms of the network lifetime and stability, our proposal is new, better and efficient when considering the energy efficiency, reliable packet transmissions, throughput and latency in the network as compared with the various existing routing techniques.

In future we intend to implement the presented scenario on human body with physical sensor nodes and performing comparison it with other existing mechanism.

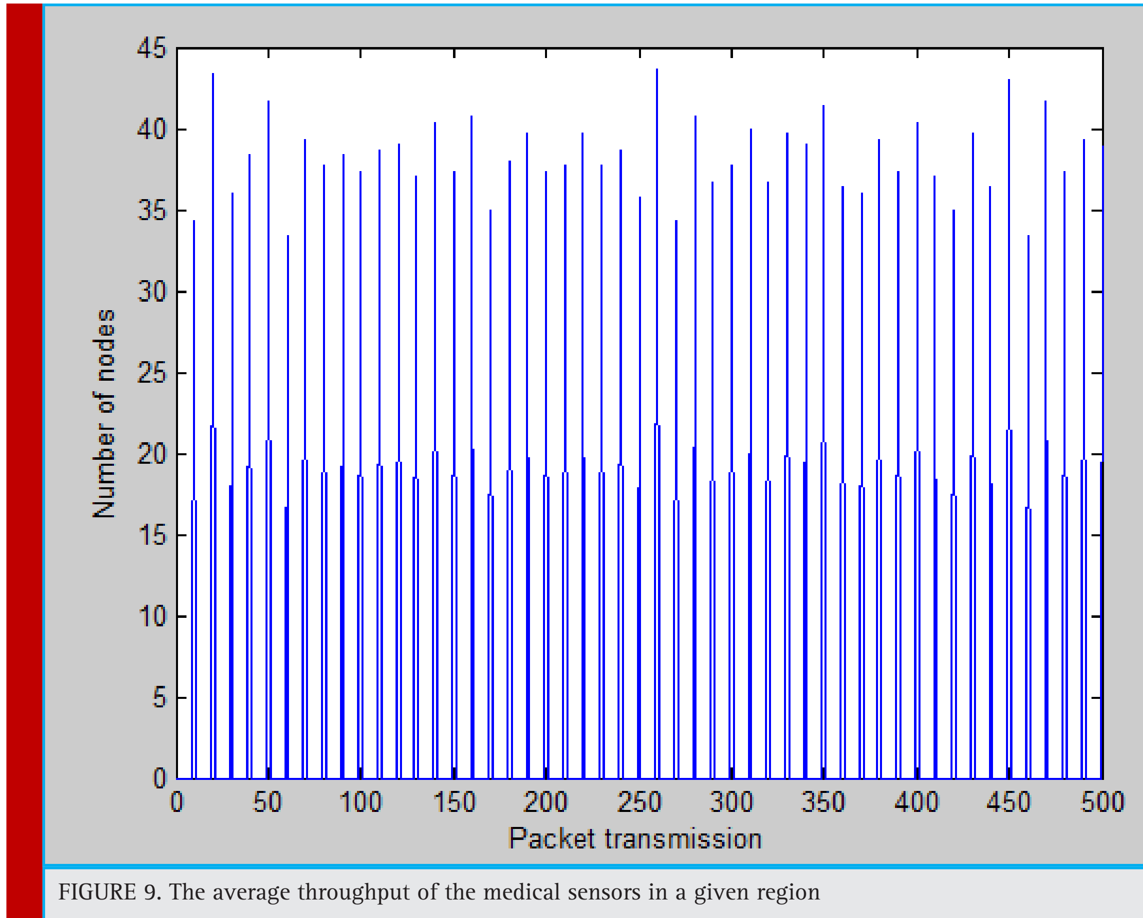

FIGURE 9. The average throughput of the medical sensors in a given region

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