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Role of Nanomedicine in Novel Corona Virus Pandemic: A Perspective

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ABSTRACT

Novel Corona virus (COVID-19) pandemic has affected numerous lives worldwide. This constant transmission of a novel coronavirus and its ability to rapidly spread from human to human has prompted scientists to develop new approaches for the treatment of COVID-19. Nanotechnology is an emerging field that can be implicated to battle against the COVID-19.Nanomedicine could be a significant theranostic means to fight against CoVs and the host cells. Nanoparticles which are embedded with viral antigens or antibodies show promising results against the SARS-CoV-2 and the re-emerging CoV. Through this communication, it is suggested that nanoparticles may play an important role at different stages of COVID-19 pathogenesis, considering their inhibition potential in the initial attachment and membrane fusion during viral entry and infected cell protein fusion. Furthermore, nano encapsulated drugs may be more efficient in activating intracellular mechanisms to cause irreversible damage to viruses and inhibition of viral transcription, translation and replication.

KEY WORDS: COVID-19, SARS-COV-2, NANOTECHNOLOGY, NANOMEDICINE.

INTRODUCTION

Nanomedicine impacts all fields of medicine and has been considered as an essential instrument for novel diagnostics, medical imaging, nano-therapeutics, vaccines and to develop biomaterials for regenerative medicine, (Tani et al 2020). Soft nanomaterials derived from polymers (polymeric nanoparticles), lipids (lipid-solid nanoparticles, nanostructured lipid carriers, liposomes), surfactants (microemulsion, nanoemulsions, liquid crystals) and proteins (protein nanoparticles) have been applied in nano medicine, especially for drug delivery. The magnitude of interactions between nanomaterial and tissues / biological molecules is the base for their use for various medical applications (Patra et al 2018). Drug-based nanoparticles have been developed for decades, and several are under clinical trials for cancer, neurodegenerative, inflammatory, cardiovascular and infectious diseases, although only few of them are approved for human use (Cui and Shi, 2019). The improvement of biopharmaceutical, pharmacokinetic and pharmacodynamics aspects of drug loading is the main tool of soft nanomaterials. Also, nanoparticles can promote specific drug targeting (passive or active targeting) and controlled drug-release rate, thereby, affecting the efficacy and safety of the treatment. Besides soft and metal nanoparticles have been applied in Nano medicine, mainly due to their various antimicrobial activities (antibacterial, antifungal, anti-parasitic and antiviral).

Due to the emergence of pathogenic bacteria resistant to antimicrobials, several studies have reported the efficacy of the nanotechnology-based antimicrobial therapy.

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Similarly, the occurrence of new viruses and their heterogeneity has also demanded innovative therapies. This way, considering specific targeting, nanotechnology opens a new avenue for antiviral therapy. The strategy of using nanoparticles to combat SARS-CoV-2 could involve mechanisms that effect the entry of the virus into the host cell until their inactivation. The blockage of the viral surface proteins may lead to virus inactivation, so targeted nanoparticles, specific to virus expressed proteins could reduce the viral internalization (Kerry et al 2019).

Metal nanoparticles have shown the ability to block viral attachment to the cell surface, leading to the inhibition of viral internalization and thereby impairing the viral replication during viral entry. Nanoparticles composed of titanium (Ti), silver (Ag), gold (Au) and zinc (Zn) have already shown results against the HIV, influenza virus, herpes simplex virus, respiratory syncytial virus, transmissible gastroenteritis virus, monkey pox virus and Zika virus .The mechanism of action is based on the nanoparticles binding onto the viral envelope or its protein, impairing the interaction with the host cell. The efficacy of the treatment is related to the size, shape and the surface charge of the nanoparticles, however, safety measures must be taken regarding the concentration to avoid cytotoxicity of host cells, (Zhao et al 2020).

Organic nanoparticles have been used for delivering antivirals such as zidovudine, acyclovir, dapivirine and efavirenz, with the aim to improve drug bioavailability and promote efficient drug delivery and targeted antiviral activity, (Devaux,2020). The main limitations of antivirals are the lack of specific targeting, resulting in cytotoxicity of the host cell, which can be addressed by organic nanoparticles. The versatility of nanoparticles makes them tunable vectors for virus targeting and specific drug delivery. Antimicrobial drugs have been tested in clinical trials for COVID-19, such as chloroquine, lopinavir, ritonavir, ribavirin and remdesivir which have demonstrated promising results against SARS-CoV-2 (Li et al,2020). Nano encapsulation of antimicrobial drugs may contribute to the development of safer treatments for COVID-19 and other viral diseases.

Although it is well-established that nanotech-based drug-delivery systems improve existing therapeutics in medicine, their application in viral diseases is underexplored and underused, as observed in the SARS-CoV-2 pandemic. Nanostructured systems can impact diagnosis, since they can improve the detection, sensitivity and increase the signal amplification specificity in polymerase chain reaction analysis; and prophylaxis as adjuvants for vaccines, as well as therapeutics for COVID-19 through the targeting of antiviral drugs,
(Uskokovic, 2020).

In conclusion, through this communication, it is suggested that nanoparticles may play an important role at different stages of COVID-19 pathogenesis, considering their inhibition potential in the initial attachment and membrane fusion during viral entry and infected cell protein fusion. Furthermore, nano encapsulated drugs may be more efficient in activating intracellular mechanisms to cause irreversible damage to viruses and inhibition of viral transcription, translation and replication.

Conflict of Interest: Nil

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Corona Viral Disease-19 Outbreak: Research Publications, Trends and Their Visualization

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ABSTRACT

Number of publications on novel coronavirus (COVID-19) is increasing rapidly; as a result it is very difficult to make an overview of the research and developments manually. There are many new studies published continuously, so it is important to know about the recent research trends or development in this exciting area. In the present communication, we have used the available bibliometric analyses of novel coronavirus to outline the growth of this topic including number of publications, keywords, co-authorships, co-occurrence, best journal and citation analyses, including the VOS viewer software. The results showed a significant growing attention on the topics of novel coronavirus outbreak (COVID-19). Analysis showed that, authors from USA and China are more active than other countries, followed by England, Italy, Canada and India. The number of publications on novel coronavirus (COVID-19) increased rapidly in April-May, 2020 as the threatening disease spread throughout the world. The analysis also revealed information on the best keywords used, co-authorships, co-occurrence, citations and the journals, among others. The study has included number of publications on novel coronavirus from around the world along with keyword analyses, co-authorship analyses, co-occurrence, and citation analyses. The analysis has also recognized the best writers and journals in the field, and it attempts to determine the future proposasl for monitoring such a vital area research.

KEY WORDS: BIBLIOMETRIC TRENDS; COVID-19; NOVEL CORONAVIRUS; SARS-COV-2; VISUALIZATION.

INTRODUCTION

COVID-19 is a disease caused by novel coronavirus (SARS-CoV-2) that can produce symptoms like common cold, cough and fever same as SARS and MERS. The novel coronavirus was originated in China during late 2019 (Gorbalenya et al., 2020; Lu et al., 2020). In March 2020, WHO announced this disease as a pandemic. The symptoms of disease appear from two to fourteen days of disclosure and it comprises temperature, cough, breathing problem, fatigue, pains, and running nose. Until now, no particular medicine or vaccine are available for COVID-19 but we can take precautions to decrease possibility of contagion (Tang et al., 2020). Social distancing and health care with oxygen treatment, fluid controlling, and antibiotics usage for microbial contaminations are suggested (Habibzadeh et al., 2020). Early detection and management can control the spread of COVID-19 disease (WHO, 2020). According to the several directives of the World Health Organization (WHO), the management of the infection could help to reduce the spread of disease (Gidengil et al., 2020). COVID-19 is a disease of international fear and its infection rate is more than the SARS (Liu et al., 2020).

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The number of persons infected and died by this disease is also more than that of the earlier severe acute respiratory syndrome, (SARS) (Mahase, 2020). Along with social distancing, quarantining of the infected persons is a key to interrupt the spread of this disease (Wang et al., 2020). If significant public transmission occurs, social distancing, closing of educational institutions, home quarantine, sanitization, and using protective tools such as facemask has been useful (Heymann et al., 2020). The COVID-19 pandemic is obviously a worldwide community health problem and has been getting increased attention to investigate and enhance the capacity of the national and international research laboratories.

The number of publications on the topic of novel coronavirus and COVID-19 are increasing continuously. Therefore, it is important to review the trends of the publications and growth of data for their application in the medicine and vaccine development. Bibliometric analysis may be helpful for this purpose, which is based on statistical analysis to explain the publication trend. A bibliometric analysis is a method to quantify science and technology and their outputs. Researchers find many definitions of the term bibliometrics (Broadus, 1987; Geisler, 2000). Generally, the term bibliometrics is used to quantitative study of publication counts or patterns of publications. Garfield had created the Science Citation Index to study the assembly of measurable elements, (Garfield, 1979). Bibliometric analyses also provide us the cognitive structure of various research fields.

Many research papers published on bibliometric methods, explain the underlying potential and limitations of these methods. There are many publications on different research topics, which can be correctly applied using these methods, but for the best of our knowledge, few elaborate statistical studies for novel coronavirus have been conducted. The present study explains the development in coronavirus publication trends, and comparison of the growth over the years along with novel coronavirus (SARS-CoV-2) and disease COVID-19. These bibliometric analyses have been used to examine the data: (1) publication counts on COVID-19 over the time, (2) countries and authors that are active on publications on COVID-19, (3) core keywords in COVID-19 publications, (4) distinguishable sub-domains related to COVID-19 research, and (5) trends in COVID-19 investigations.

MATERIAL AND METHODS

The efforts of relating bibliometric analyses to publications have demonstrated that they are extremely informative and valuable in relating communication and providing necessary scientific information. The hypothesis of bibliometric is that research publications offer suggestion of the subject areaa. Furthermore, academic publications frequently comprise a number of keywords that define these vital areas of various subjects. Analysis of networks and links can deliver future visions and workings in the related areas (Castells, 1996; Latour, 2005).

Law of distribution: There are two main laws of distribution for bibliometric procedures viz. Bradford's and Lotka's law of frequency distribution. According to Bradford distribution law, the number of journals in a subject area may distributed in three sets, each covering an identical publication count (Garfield, 1980; Vickery, 1948). Lotka law states that the number of author who published n number of publication is about 1/n2 of those published one publication (Lotka, 1926). This law also explain that half number of publications in one subject published by a very few writers which is equal to the square root of total number of writers who published in that subject (De et al., 1965; Price et al., 1961).

Choice of the data set: To know about research field, keywords are very useful. In this study, we make an overview and developments in publications on coronavirus and COVID-19. Author keywords are useful choice of terms by person and can change from one publication to other. Different writers used different keywords to specify coronavirus as a main topic in their publications. Along with other measures, we chose the data set Web of Science (Core Collection). This research was started in May 2020, and data are updated on 1st June 2020. Table 1 compares the number of publications on two data sets that is PubMed and Web of Science (WoS). The data from WoS (core collection) was recovered on 1st June 2020 with following specifications:

Topic: COVID -19Timespan: All years, Indexes: SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI.

Extra information was retrieved from PubMed to compare number of publications between the dataset and other bases. The supplementary data was taken from PubMed with following search term: (("COVID-19"[MeSH Terms] OR "COVID-19"[All Fields]), PubMed extended this search term:

COVID-19"[All Fields] OR "COVID-2019"[All Fields] OR "severe acute respiratory syndrome coronavirus 2"[Supplementary Concept] OR "severe acute respiratory syndrome coronavirus 2"[All Fields] OR "2019-nCoV"[All Fields] OR "SARS-CoV-2"[All Fields] OR "2019nCoV"[All Fields] OR ("Wuhan"[All Fields] AND ("coronavirus"[MeSH Terms] OR "coronavirus" [All Fields])) AND (2019/12[PDAT] OR 2020[PDAT]))

Bibliometric procedures: Bibliometric analyses comprise number of publications, citation analysis, and keyword analysis and so on. Number of publication specifies the size of scientific production and efficiency in a given field, so it considered as a quantification of review procedure (Melkers, 1993). In this analysis, we made publication count for years, type of publications, countries, authors, and keywords. Keyword analysis includes co-occurrences of keywords in different papers, where in keywords allotted. These findings give overview of publication trends (Ellegaard et al., 2015; Melkers, 1993).

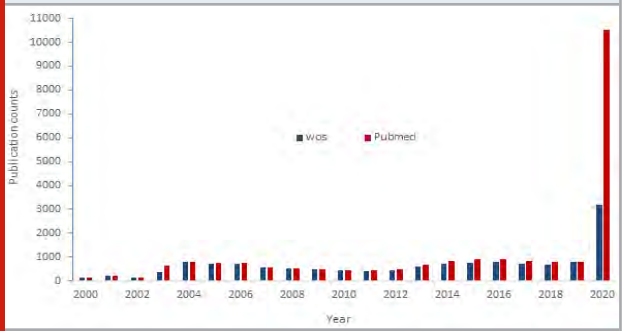
Software and pre-processing: There is a lot of software existing for bibliometric work. We selected VOSviewer software by Nic Jan (Van and Nic, 2005). It is freely available and accessible user-friendly software; commonly used for making and envisioning bibliometric graph. It allows distinctive bibliometric methods such as keyword, citation, co-authorship, co-occurrence, and many more. We did some pre-processing in Excel. To clean up the data the thesaurus option available in VOSviewer software was used.

RESULTS AND DISCUSSION

The number of publications provides understanding of the up-to-date research completed in the subject. We made several analyses associated to publications counts, types of publication, states, papers, writers, keywords, citations and co-occurrence; and represent the results under allied sub-titles.

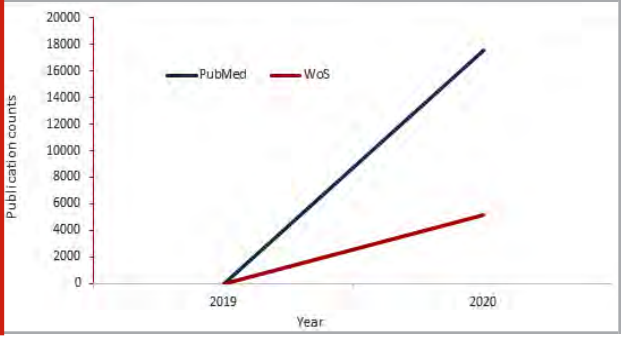
Tendencies of publications on coronavirus and novel coronavirus/COVID-19: The publication counts signify the area of scientific research. It proved by many researchers that the scientific publishing is increasing continuously with high rates in different fields (Larsen and Von, 2010; Price and De, 1963). The numbers of publication on coronavirus are also increasing continuously. Table 1 compares the number of publications on two data set that is PubMed and WoS. The main data for this analysis recovered on 1 June 2020 from WoS (core collection). The dataset suggests sharp growth of publication counts on coronavirus in year 2020, a supporting data also retrieved from PubMed on the same day for the comparison in between two data set. The retrieved data showed that the publication on coronavirus in PubMed started since 1949; but in WoS, it started from 1968 with very few publications. The significant publications found in both data set started from 2000 onwards. Publication trends of growth and decay since 2000 to 2019 in both PubMed and WoS data set is near about same as shown in figure 1.

Figure 1: Yearly number of publication on coronavirus in PubMed and WoS database



However, in year 2020 there has been a sharp increase in PubMed dataset in comparison to WoS. The publication counts on the topic coronavirus during the year 2020 in PubMed is 10527, while in WoS (core collection) only 3198. Decay in publications are reported in year 2010 in both dataset with 456 in PubMed and 458 in WoS, which represents same trends in both dataset. In current data counts, there is big difference between PubMed and WoS that may be due to selection of only core collection database of WoS. Figure 2 represents number of publication on COVID-19 since 2019 in both data sets. Since starting in 2019, PubMed contains only nine publications with zero in WoS. However, in 2020 there was a sharp increase in publications in which PubMed covered 17524 papers, along with 5210 in WoS.

Figure 2: Number of publication on COVID-19



Publication and support types: There are many types of publications for example journal article, review, editorial, case report, clinical analysis, research support and many more. We included all types of research provision from our foremost results and studied distinctly. Journal article is on the top of publication type with a count of 1837 followed by editorial material (1583), letters (1002), reviews (450), and news items (293) as shown in figure 3.

Figure 3: Publication types with counts

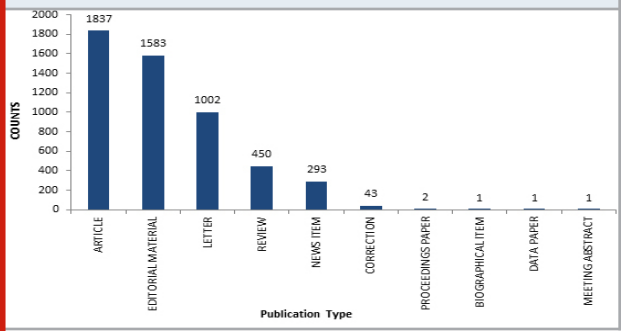
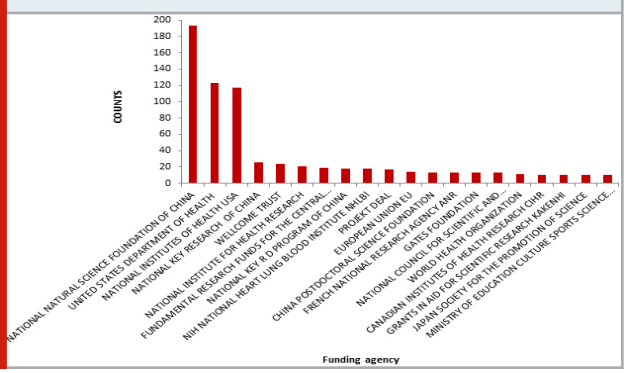


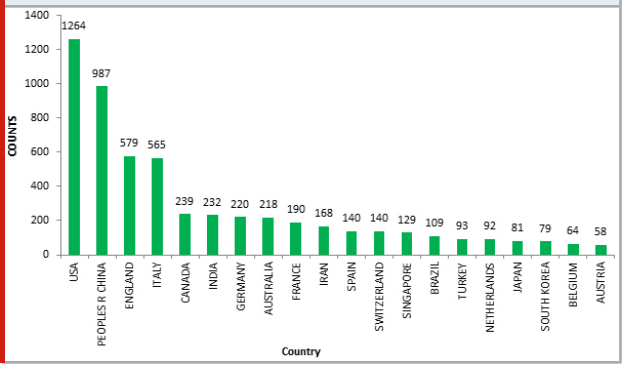
Figure 4: Funding trends for COVID-19 in 2020



Regarding funding agencies, which support COVID-19 research, National Natural Science Foundation of China is on the top position with 193 records followed by United States Department of Health Services, National Institute of Health USA, National Key Research and Welcome Trust. Figure 4 represents top 20 research funding agencies around the world.

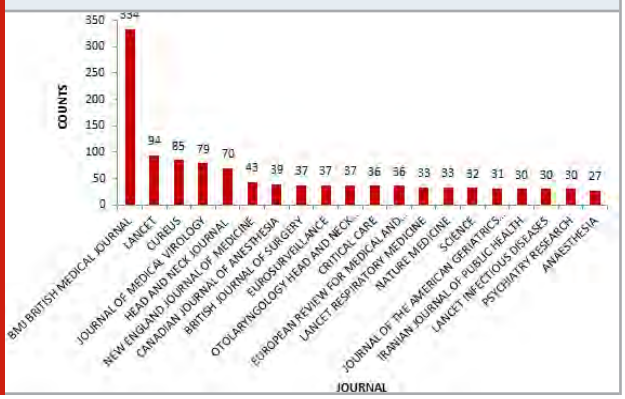
Languages and countries: In analysis of language of publication, we find that the number of publication in English are 95.29%, followed by German (1.55%), Spanish (1.42%), and French (0.40%). The publication counts in other languages are very low. For analysis of the country of publication, we used country evidence from the field 'place of publication'. USA is on the top position with 24.26% of publications out of 5210 fallowed by China with 18.9%, England 11.11 %, Italy 10.8% and Canada 4.58%. Top 20 countries with publication counts reported in figure 5.

Figure 5: Top 20 countries with number of publication counts



Journal titles: The British Medical Journal comprises highest number of publications (334) which is 6.4% of total publications on COVID-19, followed by Lancet (94) and Cureus (85). Figure 6 represents the topmost 20 journals publishing the research related to novel coronavirus and its disease COVID-19. Most of the publications are from British Medical Journal and very few are from other journal that supported by Bradford's law.

Figure 6: Top 20 journals for COVID-19 publications



Key authors: In association with Lotka's law, the results suggested opposite relationship between writers and publication counts by each writer. At one side, many authors (15757) published only one paper and on the other side a very few authors (8) have published more than 20 papers each (Figures 7). In publications, the leading author is written first, and other authors written in decreasing order of their assistances. Normally the first authors have important role in the publication of the paper. We found Mahase Elisabeth as the top writer in the dataset with 44 publications. Mahase, Elisabeth published as a single author all 44 publications; we examined many core papers by Mahase (Mahase, 2020a; 2020b; 2020c; 2020d).

Figure 7: More authors with few publications-many authors contributes only one or few publications on the mentioned topic.

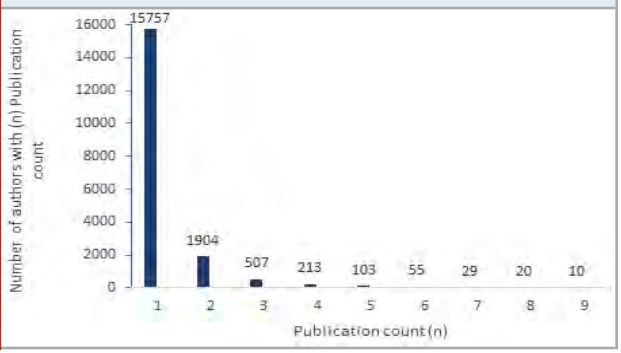
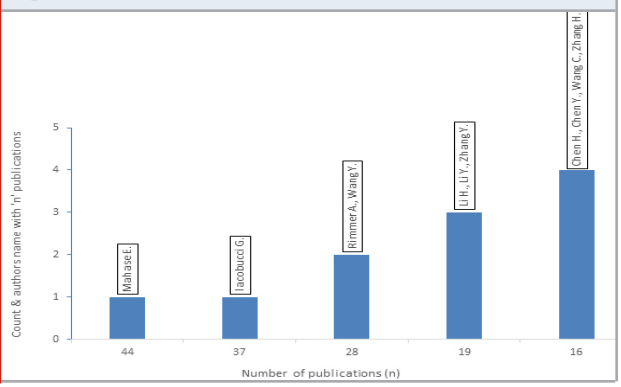


Figure 8: Few authors with more publications-small number of authors contribute many publications on the topic



Iacobuci Gareth is second highest author with 37 publications as a single author followed by dual author publications by Rimmer Abi and Wang Y, which counted to 28 articles (Figures 8). Figures 7 and 8 represent the inverse correlation as well. To find of relationships between authors, we conducted co-authorship analysis by using VOSviewer software (www.vosviewer.com). VOSviewer generates plots and picture of data based on refined grouping (Van and Waltman, 2011; Van et al., 2010). During analysis, the minimum number of papers for an author set to three and the documents that have more than 25 authors omitted. Analysis results stated that there are total 19132 authors; however, only 525

authors have co-authorship links. The co-authorship links have 17 different groups that are calculated and pictured by software as presented in figure 9. The groups or clusters are discriminated by different colors. These clusters contain minimum five (cluster 17) to maximum 27 (cluster 1) authors.

Figure 9: Co-authorship analyses with authors. The clusters made by the VOSviewer system. Colors specify cluster of related terms.

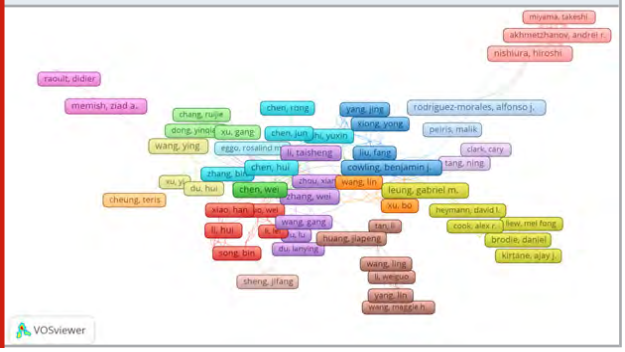


Figure 10: Co-authorship analyses with countries. The clusters made by the VOSviewer system. Colors specify cluster of related terms.

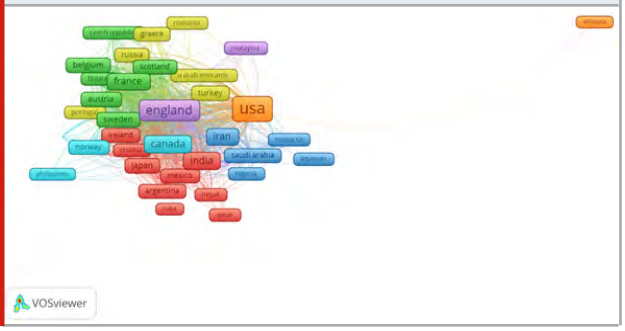
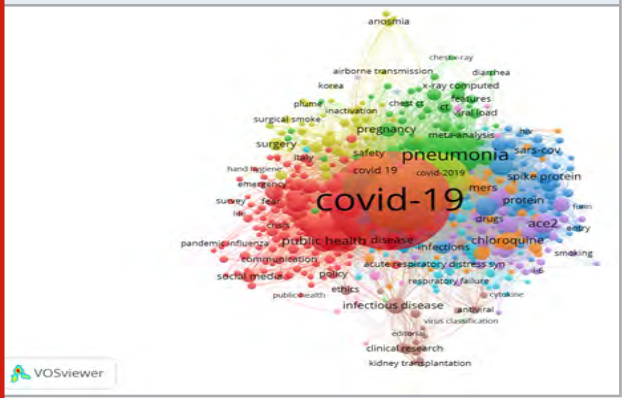


Figure 11: Co-occurrence study of all keywords

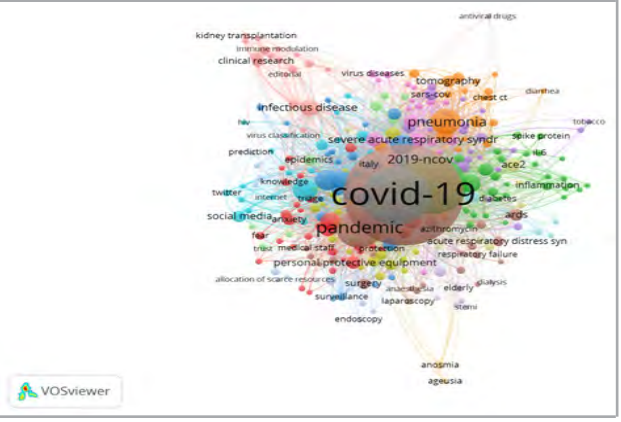


Co-authorship analyses with countries: To analyze co-authorship with countries, we used same procedure as mentioned in previous section by using VOSviewer. During analysis, the minimum number of document of a country was set to five and maximum number of countries for one document set to 25. Result shows that out of 125 countries only 69 countries have co-authorship links. The co-authorship links have seven different groups that are calculated and visualized by software presented in figure 10. The groups or clusters are discriminated by different colors. These clusters contain minimum two (cluster 7) to maximum 21 (cluster 1) countries.

Keyword analysis: WoS database contains three types of keywords viz. all keywords, author keywords, and keywords plus. This study consists of an investigation of all types of keywords. Table 2 represents top 10 keywords with frequency and total link strength. Covid-19 is most frequently used keyword with frequency of 1694.

Co-occurrence study of all keywords: The co-occurrences study of keyword is a suitable way to find the core topics of publications. To find co-occurrences analysis, we used VOSviewer tool and prepared two types of analyses such as 'all keywords' and 'author keyword'. By the thesaurus option, pre-processing and clean-up data conducted. During study, the lowest frequency of keywords set to '5', the device then designed the total co-occurrence with other keywords. There are total 6572 keywords, and 519 keywords encounter the threshold. We chose top 519 keywords to include in the studies and visualizations. Figure 11 shows the network visualization of all keywords. These keywords grouped in to 14 clusters. Cluster 1 has 143 items. The topmost keyword COVID-19 is in cluster 1 with total link strength of 6913. The top five keywords are COVID-19, coronavirus, SARS-COV-2, pneumonia and SARS.

Figure 12: Co-occurrence of author keywords.



Co-occurrence study of author keywords: In comparison to all keywords, in this segment we emphases on author keywords. The same methodology we used as mentioned in previous section. The minimum number of occurrences was set to five. There are total 4843 keywords and 305 met onset. We selected highest 305 keywords with utmost total bond power. At this time also the terms "COVID-19" is the core keyword by the authors. Seventeen clusters obtained by analysis and represented in figure 12. In calculation to co-occurrence analyses, it provides information to recognize the new keywords that emerges by the authors. Co-occurrence study of author keywords

with density visualization presented in figure 13. Colors indicates density of the keyword COVID-19 which is the densest keyword.

Figure 13: Co-occurrence analysis of author keywords with density visualization

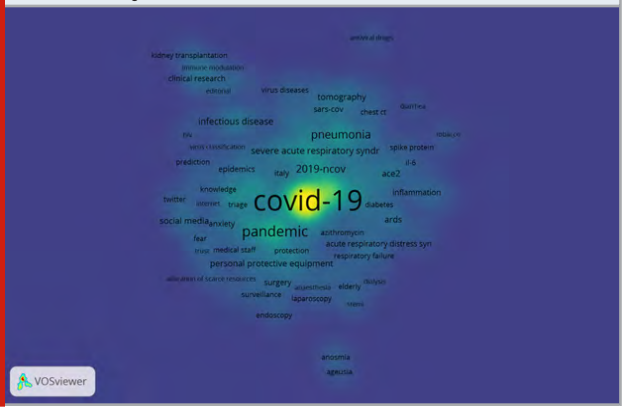


Figure 14: Citation analyses of documents. The clusters made by the VOSviewer software. Colors indicate cluster of related terms.

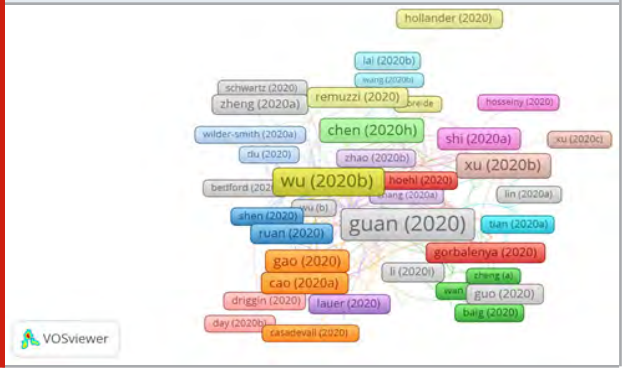
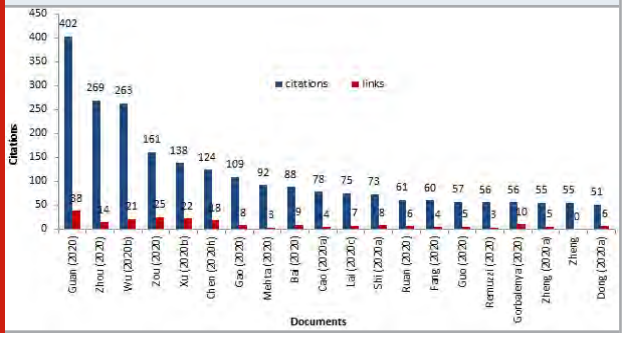


Figure 15: Top 20 documents with their citations and links



Citation analysis of documents: In this segment, we analyzed documents citation. The minimum number of occurrences fixed to five. Out of 5210 cited documents, 378 seen as threshold. The largest set of connected items consists of 271 items. For each 271 document, the number of citation link calculated and displayed in visualization. Total clusters formed was twenty-eight as represented in figure 14. Cluster 1 contains 16 items and the last cluster 28 have only four items. The documents with decreasing order of citation presented in figure 15. According to citation analyses, the result suggested that the top five documents are from China based authors.

Figure 16: Co-citation analyses of reference. The clusters formed by the VOSviewer algorithm. Colors indicate cluster of related terms.

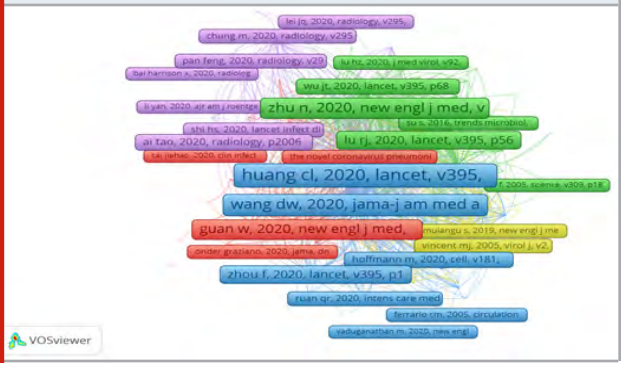


Table 1. Publications in WoS and PubMed on 1 June 2020.

Search Term	Number of publications	
	WoS	PubMed
Coronavirus	16811	25765
COVID-19	5210	17533
SARS	14268	14855
MERS	25242	19420

Table 2. Topmost ten keywords with their frequency

Keyword	Frequency	Total link strength
COVID-19	1694	69
Coronavirus	686	3466
SARS-COV-2	562	2979
Pneumonia	263	1560
SARS	244	1636
Pandemic	243	1141
Infection	151	913
China	134	697
Outbreak	133	839
Acute respiratory syndrome	117	737

Co-citation analyses of references: For co-citation analysis, minimum number of cited reference was set to 20. Out of 50176 cited references, the threshold value obtained for 304. For each of 304 cited references, the total strength of co-citation links with other cited reference calculated and visualized in figure 16. All items grouped in to five clusters viz. Cluster one (103 items), cluster two (76 items), cluster three (55 items), cluster four (36 items), and cluster five (34 items). The reference with most frequent co-citation "Huang CL, 2020, Lancet,

v395, p497, doi 10.1016/s0140-6736(20)30183-5" is in cluster 3 with total link strength of 7377. Table 3 listed twenty topmost cited reference with their total link strength. From co-citation analyses, we can conclude that the documents with highest number of citations are from China based authors.

Table 3. Topmost twenty cited reference with total link strength.

Rank	Cited reference	Citations	Total link strength
1	Huang CL, 2020, Lancet, v395, p497, doi 10.1016/s0140-6736(20)30183-5	845	7377
2	Wang DW, 2020, JAMA-J Am Med Assoc, v323, p1061, doi 10.1001/jama.2020.1585	546	4952
3	Chen NS, 2020, Lancet, v395, p507, doi 10.1016/s0140-6736(20)30211-7	487	5034
4	Zhu N, 2020, New Engl J Med, v382, p727, doi 10.1056/nejmoa2001017	452	3935
5	Guan W, 2020, New Engl J Med, v382, p1708, doi 10.1056/nejmoa2002032	360	3119
6	Zhou P, 2020, Nature, v579, p270, doi 10.1038/s41586-020-2012-7	299	3480
7	Chan JFW, 2020, Lancet, v395, p514, doi 10.1016/s0140-6736(20)30154-9	282	3299
8	Lu RJ, 2020, Lancet, v395, p565, doi 10.1016/s0140-6736(20)30251-8	266	3151
9	Zhou F, 2020, Lancet, v395, p1054, doi 10.1016/s0140-6736(20)30566-3	252	1993
10	Wu ZY, 2020, JAMA-J Am Med Assoc, v323, p1239, doi [10.1001/ jama.2020.264810.1001/jama.2020.2648 10.1001/jama.2020.2648]	245	1941
11	Li Q, 2020, New Engl J Med, v382, p1199, doi 10.1056/nejmoa2001316	227	2128
12	Holshue ML, 2020, New Engl J Med, v382, p929, doi 10.1056/nejmoa2001191	203	2620
13	Wang ML, 2020, Cell Res, v30, p269, doi 10.1038/s41422-020-0282-0	177	2446
14	Zou LR, 2020, New Engl J Med, v382, p1177, doi 10.1056/nejmc2001737	148	1398
15	Rothe C, 2020, New Engl J Med, v382, p970, doi 10.1056/nejmc2001468	145	1697
16	Van Doremalen N, 2020, New Engl J Med, v382, p1564, doi 10.1056/nejmc2004973	141	1157
17	Li Qun, 2020, New Engl J Med, v382, p1199, doi 10.1056/nejmoa2001316	131	1257
18	Xu Z, 2020, Lancet Resp Med, v8, p420, doi 10.1016/s2213-2600(20)30076-x	128	1514
19	Ai TAO, 2020, Radiology, p200642, doi 10.1148/radiol.2020200642	124	1128
20	WHO, 2020, Cor Dis 2019 COVID-19	119	789

CONCLUSION

These analyses provide a review of publications on the subject COVID-19. The study demonstrated stable progress in correlated publications, with a greater speed of progress in current year. The study determined total numbers of publications country wise and reported that the authors from USA influence the publications; fallowed by China, England, Italy, Canada and India. The publication supremacy proved by USA based author in this field and putting it as a top dealer to the worldwide. The top journals in which the articles published are British Medical Journal followed by Lancet, Cureus, and Journal of Medical Virology. In analyses of publication types, Article is on the top position fallowed by Editorial material, Letter and Review papers. Regarding research-funding agency, National Natural Science Foundation of China is at the top position is followed by United States Department of Health Services. Along with best authors in related field, the study also suggested that there are few authors with many publications but more authors with only one publication. These findings fallows distribution law of bibliometrics. The author with highest publication count is Mahase Elisabeth.

In a co-occurrence study, the best five 'all keywords' are COVID-19, coronavirus, SARS-COV-2, pneumonia and SARS. In analysis of 'author keywords', the top five keywords include COVID-19, coronavirus, SARS-COV-2, pandemic and 2019-nCOV. The top three keywords (COVID-19, Coronavirus, SARS-COV-2) are the same in both analyses that is in 'all keywords' and 'author keywords'. In density visualization also, we found that the keywords COVID-19 is a most arising keyword. Furthermore, the outcome of citation analysis of document and co-citation of reference represents that the publication by China based authors are highest cited reference. These results revealed growth of publications and delivered bibliometric assessment of COVID-19 and allied research, which can be helpful for future research and development in the field. The worldwide researchers working especially on COVID-19 pandemic will be benefited by this updated knowledge on related field.

Conflict of interest: The author declares no conflict of interest.

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Epidemiological Studies on Outbreaks of Severe Acute Respiratory Syndrome with Special Reference to the COVID-19 Pandemic

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ABSTRACT

The corona virus disease (COVID-19) which has been declared a pandemic by the World Health Organization (WHO) in March 2020, is a major concern at present, with 11,635,939 cases and 539,026 deaths worldwide, as on 08 July 2020. The SARS CoV-2 infection had emerged in Wuhan, Hubei Province, China and is likely to have originated from the zoonotic coronaviruses, WHO has initiated an investigation on the zoonotic source of disease, to ascertain how the disease jumped between animals and humans. Scientific community across the globe has been conducting research based on the knowledge from previous outbreaks of Severe Acute Respiratory Syndrome (SARS) in 2002-2003 and Middle East Respiratory Syndrome (MERS) in 2012, caused by other corona viruses, and other similar epidemics to speed up the scientific discoveries and implement solutions. There have been new revelations in this duration of six months in relation to its mode of transmission, efficacy of drugs, development of vaccines, methods of testing and the spread of disease.

The research for potential therapeutics as well as combination therapies are under different stages of trials involving about 5500 patients in 39 countries, as on 01 July 2020 . There is no panacea for the treatment of Coronavirus disease, and the current treatment plan is largely symptomatic, which is mostly a combination of classical and compassionate therapeutics. Considering the non-availability of specific therapeutics and vaccines for disease control, and with the increasing knowledge on the varying effects it generates post recovery, it is largely accepted that prevention is paramount. The present review paper aims at providing a comprehensive understanding on origin, transmission, testing, prevention, therapeutics, to provide a guideline for effective planning for controlling COVID-19 spread by suppressing transmission and preventing associated illness and death.

KEY WORDS: CORONA VIRUS, COVID-19, EPIDEMIOLOGY, PANDEMIC, PERSPECTIVES.

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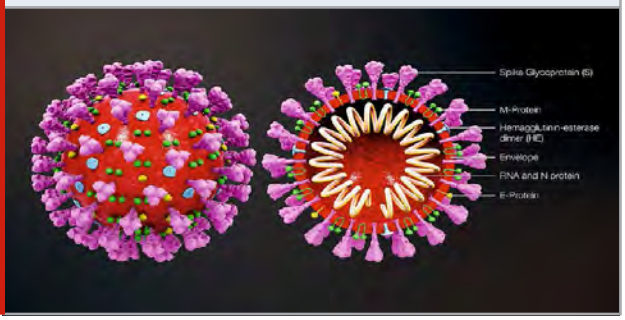
INTRODUCTION

Corona viruses belong to the family Coronaviridae in the order Nidovirales. They are enveloped positive sense RNA viruses ranging from 80 nm to 140 nm in diameter (Cheng et al., 2007). The spike like projections on the outer surface of the virus give it a crown like appearance under the electron microscope, hence the name corona virus. The structure of coronavirus with spike glycoproteins (S), membrane (M) protein, hemagglutinin-esterase (HE) and the envelope (E) protein located in the virus envelope is illustrated in Figure 1 (Jin et al., 2020). The length of RNA of these viruses ranges from 26-32 kb (Su et al., 2016). There are four subgroups of corona virus namely alpha (α), beta (β), gamma (γ) and delta (δ) (Woo et al., 2009, Cui et al., 2019). The corona viruses mainly cause Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), Acute Respiratory Distress Syndrome (ARDS) and novel corona virus disease of 2019 (COVID-19). All these lead to serious respiratory illness such as pneumonia and lung failure. A comparison of features of SARS-CoV, MERS-CoV and SARS-CoV-2 outbreaks are summarized in Table 1.

Figure 1: Coronavirus structure: Coronavirus is an enveloped, non-segmented, positive-sense single-stranded RNA virus with a genome size of approximately 26–32 kb. The genome RNA is complexed with the N protein to form a helical case within the viral membrane. The spike glycoproteins (S), membrane (M) protein, hemagglutinin-esterase (HE) and the envelope (E) protein are located in the virus envelope (Jin et al., 2020). (Image courtesy https://www.scientificanimations.com/ coronavirus-symptoms-and-prevention-explained-through-medical-animation/) .



Corona virus family was thought to infect animals before the SARS outbreak in 2002 in China (Zhong et al., 2003). Later on, a similar disease appeared in Middle East countries (MERS) in 2012 (Wang et al., 2013). The present epidemic started during December 2019 and within a short time of three months it converted into a pandemic. The World Health Organization (WHO) on 11 March 2020, declared COVID-19 a pandemic, pointing to over 118,000 cases of the corona virus illness in over 110 countries and territories around the world and the sustained risk of further global spread (WHO media briefing on COVID-19, 11 March 2020). Since then, the number of cases have increased exponentially spreading in 216 countries. Globally, as on 08th July 2020, 11,635,939 confirmed cases of COVID-19, including 539,026 deaths, were reported to WHO (WHO Corona virus Disease Dashboard). The etiological agent involved in the present outbreak belongs to the beta group of corona virus.

This is the same group of virus that was involved in SARS in Guangdong province of China in 2003 with increased infectivity and mortality probably due to high transmission rate because of genetic recombination at S protein in the receptor-binding domain (RBD) (Shereen et al., 2020). The Corona Virus Study Group (CSG) of the International Committee on Taxonomy of Viruses, which is responsible for developing the official classification of viruses and taxonomy of the Coronaviridae family, assessed the novelty of the human pathogen and tentatively named it 2019-nCoV. Based on phylogeny, taxonomy, and established practice, the CSG designated it as Severe Acute Respiratory Syndrome Corona Virus 2, SARS-CoV-2, (Gorbalenya et al., 2020). The World Health Organization (WHO) officially changed the name of the disease to Corona Virus Disease 2019 (COVID-19) from 2019 novel Corona Virus (2019-nCoV) infection, on 11 February 2020 (WHO, 11 Feb 2020).

ORIGIN OF SARS-CoV, MERS-CoV AND SARS-CoV-2

i) SARS-CoV: SARS-CoV (Severe Acute Respiratory Syndrome – Corona Virus) was identified as causative agent in an outbreak in Guangdong, China, during 2003 (Xu et al., 2004). This virus was identified as a member of beta corona virus subgroup and was given the name as SARS- CoV (Shereen et al., 2020, Zhumla et al., 2016). The affected population exhibited pneumonia with diffused alveolar injury that leads to Acute Respiratory Distress Syndrome (ARDS). During this outbreak which had affected 30 countries, a total of 8098 people were infected and there were 774 (9.7%) deaths (WHO emergencies preparedness response, 21.4.2004).

ii) MERS CoV: A coronavirus (CoV) that causes a severe lower respiratory tract infection in humans, emerged in the Middle East region in 2012, which was later named as Middle East Respiratory Syndrome Corona Virus (MERS-CoV). MERS-CoV was initially isolated from a 60-year-old Saudi patient in September 2012 (Zaki et al., 2012). The MERS-CoV was also identified as a beta corona virus with a different phylogeny. MERS caused infection to upper respiratory tract initially leading to severe respiratory disease, finally leading to pneumonia, ARDS as SARS and causing death (Memish et al., 2013). Until 2020, 2,468 cases and 851 fatalities had been reported globally (Khan et al., 2020).

iii) SARS-CoV-2: In December 2019, the Chinese government informed WHO about severe pneumonia caused by an unknown causative agent. Based on clinical manifestations, blood tests, and chest radiographs, this disease was diagnosed as virus-induced pneumonia by clinicians. The origin was considered to be human seafood market in Wuhan city of China where live animals like bats, frogs, birds, rabbits, marmots, snakes etc are sold. On January 12, 2020, National Health Commission of China declared it as epidemic (Wang et

al., 2020). It was presumed that the people who visited seafood market and/or consumed infected birds or animals were infected with pneumonia caused by the novel etiological agent. Later the same was spread from human to human through respiratory activities viz. coughing, sneezing. The respiratory droplets generated, when inhaled through the nose or mouth, can lead to human to human transmission (Li et al., 2020, Parry et al., 2020, Phan et al., 2020, Riou et al., 2020).

EPIDEMIOLOGY

Reservoirs and transmission: In order to understand the spread and control of any disease the first step is to confirm about the origin of the causative agent, its primary reservoir, or intermediate carriers from where the infection may have spread to humans. Based on the history and data collected from infected people at the starting point of outbreak at China it was confirmed that the disease is of zoonotic origin. The reservoirs reported for corona viruses are mammals and birds (Bassetti et al., Ji et al., 2020). The present COVID -19 has 88% genomic sequence similarity with the bat derived SARS (Lu R, et al., Wan et al., 2020).

Table 1. SARS CoV, MERS CoV and SARS CoV2 Outbreaks at a Glance

S.No.	Features	SARS CoV	MERS CoV	SARS CoV2
1	Outbreaks	November 2002 (Zhong et al., 2003)	September 2012 (Zaki et al., 2012)	December 2019 (Wang C et.al., 2020)
2.	Location	Guangdong, China (Zhong et al., 2003)	Saudi Arabia (Zaki et al., 2012)	Wuhan, China (Wang C et al., 2020)
3.	Controlled	July 2003 (WHO preparedness, 2004)	Continues with low incidence rate	Continues with high incidence rate
4.	Host	Bat, Palm civets, Raccon dogs (Cheng et al., 2007)	Dromedary camels (Khan et al., 2020)	Bat, pangolin (Lu R et al., 2020)
5.	Number of countries affected	29 (Zumla et al., 2016)	27	216: as on June 01, 2020 (WHO: Coronavirus Disease, COVID-19, Dashboard https://covid19.who.int
6.	Symptoms	Fever, malaise, headache, diarrhoea, shivering, cough, shortness of breath (Cheng et al., 2007)	Fever, cough, shortness of breath (Zaki et al., 2012 Memish et al., 2013)	Fever, malaise, dry cough, shortness of breath and respiratory distress. (Wang C et.al. 2020)
7.	Morbidity	8098 (Shereen et al., 2020 Zumla et al., 2016)	2468 (Khan et al., 2020)	11,635,939: as on 08 July 2020 (WHO: Coronavirus Disease, COVID-19, Dashboard https://covid19.who.int
8.	Mortality	776 (9.6%) (Shereen et al., 2020)	851 (34.5%) (Khan et al., 2020)	539,026 (4.63%) : as on 08 July 2020 (WHO: Coronavirus Disease,COVID- 19, Dashboard https://covid19.who.int
9.	Recovery	7322 (Shereen et al., 2020)	1617	6,488,079: as on 08 July 2020 (Johns Hopkins University: Corona Resource Center
10.	Causative agent	SARS-CoV (β group) (Shereen et al., 2020 Zumla et al., 2016)	MERS-CoV (β group) (Zaki et al., 2012)	https://coronavirus.jhu .edu/map.html) SARS-CoV2 (β group) (Khan et al., 2020)

The cause of transmission, in cases where patients who did not visit seafood market but acquired infection, was as a result of human-human interaction through respiratory activities (Carlos et al., 2020, Wu et al., 2020). The chances of hospital acquired infection at secondary or tertiary stage can't be neglected. Transfer of virus from pregnant woman to newborn is not yet reported. Migration of infected people from one city to other and one country to other is the possible way of transfer of COVID-19, globally. Importantly, during the initial stage of the outbreak no screening of migrants was done and

even if the migrants were screened later, the screening was limited to measuring body temperature which can only detect people with symptoms and not those who are asymptomatic. However, later on many countries adopted the strategy of quarantine of migrants for about 14 days and observed them for the onset of symptoms, but it was too late by then.

Basic Reproductive Number (R_0): COVID-19 is a highly infectious disease, with a basic reproductive number (R_0) estimates ranging from 1.4 to 3.5 (Chatterjee et al., 2020). It is important to emphasize on the reduction of R_0 values for controlling the outbreak size. Between December 10, 2019 and January 4, 2020, analysis of the growth rate of the epidemic gave a R_0 of 2.2, meaning each patient was spreading the infection to 2.2 other individuals (Li Q, et al., 2020). The early WHO estimate of R_0 was 1.4 to 2.5 (Chatterjee et al., 2020). Preliminary studies, conducted at the beginning of the outbreak, reported higher estimates of R_0, in the range of 2.24-3.58 (Zhao et al., 2020). Other estimates place it in the range of 2.0-3.1 and at 3.11 (95% confidence interval, CI, 2.39- 4.13) (Majumder et al., 2020, Read et al., 2020). In a data-driven analysis of the probable outbreak size on the Diamond Princess cruise ship, distribution of R_0 of COVID-19 was about 2.28 (Zhang et al., 2020). Studies related to transmissibility of COVID-19 indicate that the human-to-human transmission is the most probable explanation for the magnitude of the on-going outbreak (Imai et al., 2020). In general, an epidemic will increase as long as R_0 is greater than 1, and control measures aim to reduce the reproductive number to less than 1 (Li Q, et al., 2020).

Incubation Period: In SARS-CoV-2 infection, the period from infection to appearance of symptoms varies. Generally, it is thought to range from 2-14 days with a mean incubation period of 5.2 days (Singhal, 2020, Jin et al., 2020). This period depends on many factors like age, sex, patient's immune status, environmental conditions, etc. The period from the onset of COVID-19 symptoms to death ranges from 6 to 41 days with a median of 14 days (Wang et al., 2020). SARS-CoV-2 infection can cause five different outcomes: asymptomatically infected persons, mild to medium cases, severe cases, critical cases, and death (Jin et al., 2020).

Receptor Interactions and Cell Entry: Human angiotensin-converting enzyme 2 (ACE2) is a functional receptor that provides a direct binding site for the S proteins of coronavirus. SARS-CoV-2 utilizes ACE2 as a cellular entry receptor. ACE2 is a type I membrane protein expressed in lung, heart, kidney, and intestine mainly associated with cardiovascular diseases (Jin et al., 2020).

Symptoms and pathogenesis: The most common symptoms are fever, cough, fatigue, headache, sputum production, haemoptysis, diarrhea, dyspnoea and lymphopenia (Carlos et al., 2020, Huang et al., 2020, Ren et al., 2020, Wang W et al., 2020, Patel et al., 2020). Other abnormal features include RNAaemia, Acute Respiratory Distress Syndrome (ARDS), cardiac injury, grand glass opacities that leads to death (Huang et al., 2020). The symptoms associated with SARS, MERS and COVID-19 are almost similar. However, people infected with COVID-19 develop gastrointestinal symptoms like diarrhea. A low percentage of SARS or MERS patients exhibited similar GI symptoms. The severity of COVID-19 infection was established with the logarithmic increase in morbidity and mortality. Started on 29 December 2019 with 05 cases and 01 death the number reached to 51,174 with 1,666 (3.25%) death cases by 16 February 2020, in China alone. The median age in these cases was 75 years (range 48-89 years) (Wang W, et al., 2020). Infected persons show higher leukocyte counts i.e. 2.9 x 109 cells/L of blood of which 70% are neutrophiles. Blood C reactive protein increases up to 16 mg/L of blood. High erythrocyte sedimentation rate and D dimer are also observed along with abnormal respiratory findings, increased level of plasma pro-inflammatory cytokines. The patient's sample shows a positive real time polymerase chain reaction that confirms COVID-19 infection (Lei et al., 2020).

The clinical manifestations of COVID-19 are heterogeneous. In a study, 20–51% of patients were reported as having at least one comorbidity, with diabetes, hypertension and other cardiovascular and cerebrovascular diseases being most common (Guan et al., 2020). Experts are now also observing that critical patients with COVID-19 show signs of blood clots which can be life threatening. Such blood clots can obstruct a blood vessel and stop blood flow, the condition known as thrombosis and when this clot travels to other organs, phenomena known as embolism occurs which is again severe enough to cause death. In patients with severe clinical features of COVID-19 infection, the proportion of patients with acute pulmonary embolus was 23% (95% CI: 15%, 33%) on pulmonary CT angiography (Grillet et al., 2020).

Prevention and Control: At present there are no potential antiviral drugs or vaccines available against COVID- 19. Hence, prevention is the only way left to control the disease. As mentioned earlier COVID-19 is a lower respiratory virus enters the human through the respiratory path. In order to prevent human-human transmission the first thing is to develop method for effective and early detection of virus. Many companies have developed the PCR based detection kits. The other important ways to prevent COVID-19 is to keep social distancing (1-3 meters) and observing hand hygiene particularly by the health care workers and family members who are in contact with patients. WHO has already released guidelines for hand hygiene, as shown in Figure 2 (WHO guidelines on Hand Hygiene in Health Care, 2009). PPE including protective mask, clothing, and glasses must be used by healthcare workers. Along with these measures, regular decontamination of surfaces should be done. It is important to disinfect inanimate surfaces in the surgery or hospitals as patients may touch and contaminate surfaces such as door handles, desks, etc. (Kampf et al., 2020).

To reduce the risk of infection with COVID-19, basic preventive measures to be followed at all times, were issued by the Ministry of Health and Family Welfare (MoHFW), Government of India, on 18 May 2020. These include: (i) maintaining physical distancing of at least one meter to be followed at all times, (ii) mandatory use of face covers/masks, (iii) practicing frequent hand washing (for at least 40-60 seconds) and use of alcohol based hand sanitizers (for at least 20 seconds), (iv) following respiratory etiquettes, which involves strict practice of covering one's mouth and nose while coughing/sneezing with a tissue/handkerchief/flexed elbow and disposing off used tissues properly. (v) self-monitoring of health by all and reporting any illness at the earliest.

Figure 2: Hand washing steps: duration of the entire procedure, 20-30 seconds (Adapted from WHO Guidelines on Hand Hygiene in Health Care, 2009)

POTENTIAL THERAPEUTICS

Potential antiviral drugs: Currently, there are no specific antiviral drugs or vaccines for the control of SARS-CoV-2. Present strategy of treatment of COVID-19 cases involves the use of broad spectrum antiviral drugs like nucleoside analogues and also HIV protease inhibitor (Lu H, et al. 2020).). Use of broad spectrum antiviral drugs like Favipiravir, Remdesivir, Ribavirin, etc., are involved in the present strategy of treatment of COVID-19 cases. Other existing drugs used for the treatment include the corticosteroid, Dexamethasone, and Camostat mesylate, the synthetic serine protease inhibitor, with anti-inflammatory, antifibrotic, and potential antiviral activities (Patel, et al., 2020). Oseltamivir, Lopinavir, Ritonavir via oral route, and Ganciclovir through intravenous route for 3-14 days are the drugs of choice (Chen et al., 2020). Other compounds like EIDD 2801 that have shown high therapeutic potential against seasonal and pandemic influenza can act as potential drugs to be considered for the treatment of COVID-19 cases (Toots et al.,2019).

It is clear that urgent research is needed to identify new chemotherapeutic drugs and develop vaccines. WHO is running an international therapeutics trial–the Solidarity trial, and as on 01 July 2020, nearly 5500 patients in 39 countries had been recruited into the trial (WHO, 2020 e). As on 07 July 2020, there are around 139 vaccines in preclinical evaluation phase and around 21 candidate vaccines in clinical evaluation phase (WHO, Draft landscape of COVID-19 candidate vaccines).

ii) Monoclonal antibodies: Tocilizumab, a potential recombinant monoclonal antibody against Interleukin 6 (IL-6) is currently under investigation for the management of ARDS in patients with COVID-19. Treatment of severe COVID-19 cases with Tocilizumab mitigates cytokine storm and averts mechanical ventilation during Acute Respiratory Distress (ARD) (Marovich et al., 2020).

iii) Convalescent plasma therapy: Convalescent plasma therapy has been used for severe respiratory tract infections including SARS and influenza A (H1N1) (Cheng et al., 2005, Hung et al., 2011). Ebola patients had also received the treatment of convalescent plasma (Kraft et al., 2015). A promising approach in combating SARS-CoV-2 during the outbreak would be to use plasma from the convalescent patients. Recently, convalescent plasma has been widely recommended to be used for COVID-19 (Li H, et al., 2020). Neutralizing antibodies (NAbs) against SARS-CoV-2 are important therapeutic agents proposed for the treatment of COVID-19 (Zhou and Zhao,2020). However, the outcomes of plasma therapy are unpredictable due to variability of sera in different patients. A patient from South Delhi was the first in India to recover after receiving treatment with plasma therapy, as reported by Jeelani and Mishra in India Today, on 25 April 2020. Indian Council of Medical Research (ICMR) and Drug Controller General of India (DGCI) are two nodal agencies to approve clinical trials for plasma therapy in India.

iv) Antithrombotic therapy: Prophylactic doses of heparin might be associated with improved survival (20%) in patients with evidence of sepsis induced coagulopathy (SIC) (Tang et al., 2020).

Diagnostic Tests: Nucleic acid testing such as real time RT-PCR is the main technique used to detect the novel coronavirus, COVID-19, and to confirm suspected cases (Corman et al., 2020). Other than the molecular-based approaches, serological antibody testing is also valuable in detection of novel corona virus infection (Meyer et al., 2014).

i) Real time RT-PCR: Real time RT-PCR has been a gold standard measure for diagnosis of COVID-19 and is the most accurate way of detecting the presence of SARS-CoV-2 in respiratory specimen (Corman et al., 2020). Various real-time RT-PCR protocols, which differ in the genes they detect, have been proposed for the diagnosis of COVID-19 (Hong et al., 2020). The primers and probes targeting specific genes of SARS-CoV-2 are used in real-time RT-PCR assays as diagnostic tests. The first open reading frames (ORF 1a and 1b), RNA-dependent RNA polymerase gene (RdRp), envelope (E), and nucleocapsid (N) have become key diagnostic targets for SARS-CoV-2 identification (Ahn et al., 2020). However, RT-PCR has certain limitations. It requires certified laboratories, expensive equipment, and trained technicians to operate. Thus, RT-PR is not scalable due to the lack of testing

equipment and consumables (Frost et al., 2020). The human and laboratory resources required in this method makes it difficult to deal with the large volumes of samples. One technique to reduce the number of tests required is the pooling of samples for analysis by RT-PCR prior to testing.

Pooled testing strategy can help accelerate the surveillance for COVID-19 identification in a community or group of people living together. Testing samples from multiple patients with a single PCR test, also known as pooled sampling, has been used previously in the early stages of the HIV epidemic when PCR costs were high (Emmanuel et al., 1988). Pooled RT-PCR testing can vastly increase testing for COVID-19. In this method multiple swab samples are pooled in a test tube and they are tested using a single RT-PCR test. If the test is negative, all the people tested are negative. If found positive, a pooled sampling exercise, can be done to trace back to the individual(s) (Narayanan et al., 2020). The researchers used mathematical analysis to explore efficient pooling strategies using this technique. They recommend the use of the pooled sample method with a binary hierarchical testing strategy for the detection of SARS-CoV-2 by RT-PCR in community surveillance. This method can enhance the capacity to test in a low-resources setting where testing kits, facilities, and personnel are scarce.

One limitation of pooling multiple RT-PCR samples is that the sensitivity of testing is reduced. To address this, it has been suggested that the number of samples being pooled be kept as low as possible to reduce dilution (Westreich et al., 2008, Muniesa et al., 2014). Based on a study conducted at DHR/ICMR Virus Research & Diagnostic Laboratory (VRDL) at King George's Medical University (KGMU), Lucknow, India, ICMR has recommended sample pooling for real-time RT-PCR screening for COVID-19 in areas or population with low prevalence of COVID-19. In areas with positivity of 2-5%, sample pooling for PCR screening has been recommended only in community survey or surveillance among asymptomatic individuals. Pooling of sample has not been recommended in areas or population with positivity rates of >5% for COVID-19 (ICMR-Information of testing strategies, 13 April 2020).

ii) Rapid Antibody Tests: Rapid and accurate detection of COVID-19 is very crucial in controlling outbreaks in the population. The limitations of RT-PCR test makes it unsuitable for use in the field for rapid and simple diagnosis and screening of patients. Rapid and simple diagnosis and screening of patients can be achieved by testing of specific antibodies of SARS CoV-2. Rapid antibody tests can detect immunoglobulin M (IgM) and IgG antibodies simultaneously against SARS CoV-2 virus in human blood within 15 minutes. The IgM-IgG combined assay is useful for the rapid screening of SARS CoV-2 carriers, symptomatic or asymptomatic, in the population (Li Z et al., 2020).

THE INDIAN SCENARIO

COVID-19 Cases: In India, the first COVID-19 case was reported in Kerala on 30 January 2020. Only 3 cases were reported till 02 March 2020, however, by 05 March 2020, 29 cases had been reported. One case was reported in an Indian who traveled back from Vienna and exposed a large number of school children in a birthday party at a city hotel (Singhal, 2020). As per the ICMR report of 10 April 2020, total of 1,61,330 samples from 1,47,034 individuals were tested, 6,872 individuals were confirmed positive in India. 764 patients recovered from the disease and there were 246 death cases (ICMR: COVID-2019 data portal).

However, within a span of about 51 days, as on 01 June 2020, India stood seventh in the world in terms of COVID-19 cases with 1,90,535 cases. The cases increased more rapidly thereafter, and India ranked third in the world as on 08 July 2020 with 7,42,417 COVID-19 cases and 20,642 deaths reported (WHO: Coronavirus Disease Dashboard) and there were 4,56,831 recovered cases (Johns Hopkins University: Corona Resource Centre). Up to 08 July 2020, 10740832 samples were tested in India (Official Updates Coronavirus – COVID-19 in India – mygov.in).

COVID-19 Testing Strategies in India: Test, track, treat is the key strategy for early detection and containment of the pandemic. To combat COVID-19, India adopted various testing strategies for tracing infected cases and creating an infrastructure to provide testing facilities and services across the country. To ramp up the testing in the country, ICMR approved of 1049 public and private laboratories for COVID-19 testing as on 01 July 2020. (ICMR portal, Testing Strategy, 01 July 2020). Along with the existing testing strategies, newer additional strategies for COVID-19 testing have been adapted. To facilitate testing at district level the government has tapped the rich resource of available TrueNat machines, the diagnostic machines used for tuberculosis diagnosis. Along with Real Time RT-PCR, the gold standard test for detecting cases of COVID-19, the TrueNAT and CBNAAT (Cartridge Based Nucleic Acid Amplification Test) systems have also been deployed for diagnosis of COVID-19. These platforms have widespread availability even at district and primary health center level as they are widely used for diagnosis of tuberculosis and other infectious diseases.

The viral lysis buffer that comes with the COVID-19 cartridges inactivates the virus and poses minimum biosafety hazard. The closed nature of these platforms and minimum sample handling further augment their safety. These features have facilitated use of these platforms at grass root level thereby increasing access to testing. Rapid Point-of-Care (PoC) Antigen Detection Test (for diagnosis along with RT-PCR) and IgG Antibody test for COVID-19 (only for surveillance and not diagnosis) are also recommended by ICMR (ICMR Advisory: Newer Additional Strategies for COVID-19 Testing, 23 June 2020).

PERSPECTIVES

Recommendations for Future: As on 08 July 2020, the novel corona virus, SARS CoV-2, has spread and badly hit about 216 countries across the globe, with 11,635,939 confirmed cases and a mortality of 539,026 cases (4.63%) (WHO: Coronavirus Disease Dashboard). Table 2 summarizes the data of COVID-19 cases in top 10 countries across the globe as on 08 July 2020 (WHO: Coronavirus Disease Dashboard, Johns Hopkins University: Corona Resource Centre). Looking to the rapid rate of transmission of the disease, measures for disease containment with lower magnitudes of loss to human life and economy need to be taken. Rapid testing methods, screening and isolation of asymptomatic patients and strict implementation of infection prevention and control measures are highly recommended for combating the situation. A few recommendations are mentioned below:

Table 2. Top Ten Countries Affected by COVID-19 as on 08 July 2020 (WHO: Coronavirus Disease (COVID-19) Dashboard), (Johns Hopkins University: Corona Resource Centre).

S.No.	Countries & Territories	Cases (WHO)	Deaths (WHO)	Recovered cases (JHU)
1	United States of America	2,923,432	129,963	9,36,476
2	Brazil	1,623,284	65,487	11,07,012
3	India	7,42,417	20,642	4,56,831
4	Russian Federation	694,230	10,494	4,71,718
5	Peru	305,703	10,772	2,00,938
6	Chile	301,019	6,434	2,68,251
7	United Kingdom	285,772	44,236	1,375
8	Mexico	261,750	31,119	2,09,437
9	Spain	251,789	28,388	1,50,376
10	Iran (Islamic Republic of)	245,688	11,931	2,09,463

Globally, as on 08 July 2020, 11.44am CEST, 11,635,939 confirmed cases of COVID-19, including 539,026 deaths, were reported to WHO

i) Adopting strategies of tracing, testing and treating cases: The approach of focusing on tracing , tracking, testing, and treating cases has been found to be successful to contain the spread of COVID-19, as in the case of Dharavai, the largest slum of Asia, located in Mumbai, India. Dharavi, spread over an area of 2.5 square kilometers and with a population of 650,000, was once declared a COVID-19 hotspot. It recorded its first COVID-19 case on April 1, and till 10 July 2020, 2,359 COVID-19 cases have been recorded in Dharavi of which 1,952 have recovered and there were 166 active cases, till date (Times of India, 11 July 2020). The Dharavi model of combating COVID-19 has been acknowledged and appreciated by the WHO. On 10 July, 2020, WHO Director-General Tedros Adhanom Ghebreyesus while addressing a virtual media briefing acknowledged the measures adopted to contain the spread of the virus in Italy, Spain, South Korea and in Dharavi, quoting- "a strong focus on community engagement and the basics of testing, tracing, isolating and treating all those that are sick is key to breaking the chains of transmission and suppressing the virus" (WHO Director-General's media briefing on COVID-19 – 10 July 2020).

ii) Use of pulse oximeter for screening: Early recognition and rapid diagnosis of COVID-19 are essential to prevent transmission and provide supportive care in a timely manner. Widespread pulse oximetry screening for COVID pneumonia could provide an early warning system for the kinds of breathing problems associated with the disease. A pulse oximeter can provide early warning of the kinds of breathing problems associated with COVID-19 pneumonia. This device when placed on a fingertip, displays: oxygen saturation and pulse rate. This was reported in New York Times on 20 April 2020, by Dr Richard Levitan, an emergency physician in Littleton, Town in New Hampshire, United States of America.

According to Dr Levitan, COVID pneumonia initially causes a form of oxygen deprivation, a "silent hypoxia," which is hard to detect. Normal oxygen saturation for most persons at sea level is 94 to 100 percent; COVID pneumonia patients have lower oxygen saturation. By detecting silent hypoxia early through a common medical device: a pulse oximeter, more asymptomatic patients who have COVID pneumonia could be identified sooner and treated effectively. Although, oximeters are not 100 percent accurate, they may be used as means for screening and early detection of silent hypoxia along with other confirmatory tests.

iii) Convalescent plasma therapy: Convalescent plasma therapy is a promising approach in combating SARS-CoV-2. Passive immunization has been successfully used to treat infectious diseases. High-quality studies and the need for adequate selection of donors with high neutralizing antibody titers should be considered (Cunningham et al., 2020). Convalescent plasma can be given to a sick people to boost their immunity. But a good number of donors must come forward after their recovery and plasma must have good amount of antibodies to help the sick.

iv) Pooled testing strategy: Pooled testing strategy for COVID-19 may benefit India. Pooling of samples can help accelerate the surveillance for COVID-19 identification in a community or group of people living together. This strategy could reduce the time, cost, and resources required and help to identify infected people in a population. Group testing can be beneficial in reducing the number of tests required to assess whether the infection rate in a population is low or high (Narayanan et al. , 2020). Testing on asymptomatic individuals

by pooled sample testing can save many test kits in particular.

v) Rapid screening and isolation of asymptomatic patients: Asymptomatic persons are thought to be potential sources of SARS- CoV-2 infection (Rothe et al., 2020) which may have caused the rapid spread of SARS-CoV-2. This asymptomatic spread may be one reason that the control strategy based on the isolation of patients has not been fully successful. Screening of asymptomatic patients and their isolation would help to prevent the random spread of infection by them. Rapid antibody tests are useful for the rapid screening of SARS CoV-2 carriers, symptomatic or asymptomatic, in the population (Li Z et al., 2020).

vi) Following infection prevention and control measures: Infection prevention and control measures as recommended by WHO (WHO interim guidance, March 2020) should be effectively followed. In fact, it should be followed in any such outbreak in future too. Standard precautions include-

Practice hand and respiratory hygiene

- Offer a medical mask to patients with suspected COVID-19 while they are in waiting/public areas or in cohorting rooms.
- Use of appropriate personal protective equipment (PPE) according to a risk assessment.
- Practice safe waste management, environmental cleaning, and sterilization of patient care equipment and linen.
- Considering all specimens collected for laboratory investigations as potentially infectious.

CONCLUSION

The COVID-19 pandemic has challenged the economic, medical and public health infrastructure of many countries across the globe. The biggest problem is to combat and curb the spread of the outbreak in the absence of vaccines and suitable antiviral agents. Social distancing, isolation and quarantine are useful measures to stay safe from this pandemic infection. It is necessary to develop drugs and vaccines against the COVID-19 infection as soon as possible. Until then, monitoring spread of COVID-19 by screening of symptomatic and asymptomatic patients and their isolation, mass testing of population by rapid testing methods and enforcement of infection prevention and control measures are highly recommended for combating the situation.

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Assessing the Needs of Health Policy Education for Medical Professionals Following Healthcare Transformation in Saudi Arabia

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ABSTRACT

Recently, certain factors have increased the importance of health policy training for medical professionals in Saudi Arabia, especially following the health care transformation initiatives. This study presents health policy course content that can be used as a foundational material for teaching health policy, and assesses needs regarding teaching health policy among medical professionals. Mixed methods, used in previous study, were used to achieve the study objectives. Pre- and post-workshop (8 workshops) questionnaires were developed to assess health policy knowledge among senior health care professionals, with a total 285 participants. A semi-structured interview with deans of medical colleges was used to assess their attitudes toward teaching health policy for medical professionals. Compared with the other groups, senior consultant physicians scored lowest (mean scores 2.75 among senior consultant physicians, 4.38 among other service professionals, and 5.75 among management professionals). However, after the workshops, knowledge levels were similar across all three groups. Also study finds agreement among medical college deans regarding the importance of teaching health policy throughout physicians` career path and disagreement among deans as to the appropriate professional level for such training. The study shows the importance of providing formal training on health policy during physicians' medical education. Thus, being an important part of society, the medical community at large should understand the societal complexities of health and thus integrating the subject of public health policy within medical curriculum program should be a welcoming step in this direction..

KEY WORDS: HEALTH POLICY TRAINING; HEALTH POLICY KNOWLEDGE; HEALTH POLICY EDUCATION.

INTRODUCTION

According to WHO, Health policy refers to decisions, plans, and actions that are undertaken to achieve specific health care goals within a society (WHO 2011). Over the years globally, the practice of medicine are increasingly been influenced by geo-political, socio-economic factors where various stakeholders including governments, private players, NGOs, International as well as transnational agencies are the principal guiding forces, (Khatana 2017; MacNeil et al., 2019). Literature review suggests that both prevention and treatment policies – which lay the basic foundation of modern medicine are impacted by these non-medical determinants. They are known to influence the behavior of population at large, thereby affecting the health outcomes. Thus, Health policy training for health care professionals has increased in importance in recent years.

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However, the manner in which health policy training needs are determined can vary (Goetz, Arora, & Press, 2010; Patel, Davis, & Lypson, 2011; Heiman, Smith, McKool, Mitchell, & Roth Bayer, 2015; Bayer et al., 2017; Dark, Pillow, & Haddock, 2018).

Most published studies have focused on the use of different training materials owing to differences in the academic level of medical professionals(Goetz et al., 2010; Greysen, Wassermann, Payne, & Mullan, 2009; Patel et al., 2011; Kidd, Cawley and Kayingo, 2016; Dark et al., 2018). In addition, there are few published studies regarding health policy education for nurses (Ellenbecker et al., 2017). However, it seems clear that health policy teaching programs are not yet sufficiently developed to fit the needs of different health care professionals. Most of the health policy concepts and analyses have been replicated directly from the developed countries without giving much thought about its impact with respect to developing nations. Very little research has been conducted to examine these prepositions from the context of these low income and middle income economies as extensive differences exist between the geo-politico-socio-economic environments (Walt et al., 2008; Patel et al., 2011; Khatana 2017; Kiendrebeogo et al 2020).

With reference to Saudi Arabia, certain influencing factors have propelled the importance of teaching health policy to healthcare professionals. The challenges associated with policy framing, meeting the targeted health outcomes, managing the undergoing major reforms which the Saudi healthcare systems are facing have immense implications for future, (Al Khamis, 2016). From a macro-level health policy perspective, the "Health in All Policies` initiative has been developed in close collaboration with the National Transformation Program, (Ministry of Health, 2017). In alignment with the Ministry of Health (MOH) Vision 2030, transformation of the health care sector in Saudi Arabia is currently underway. Transformation of the role of the MOH involves a change from having the responsibility for planning, financing, regulation, and provision and supervision of publicly financed health care services to a focus on systems governance and stewardship. The new reforms will lead to separation of the MOH from payer and provider functions (Ministry of Health, 2017, 2018).

From a micro-level health policy perspective, part of the transformation of the MOH with respect to providers involves the creation of a holding company responsible for managing exiting MOH providers. The holding company will in turn manage the establishment of a total 20 geographic clusters and will support the development of these autonomous clusters (Ministry of Health, 2018). The strategic planning of the MOH involves decentralizing health services and increasing autonomy via these clusters. These autonomous clusters are expected to increase the efficiency of medical and managerial functions, achieve financial and administrative flexibility through adopting a direct budget strategy, apply quality assurance programs, and simplify the contractual process with qualified health professionals (Ministry of Health, 2018).

The MOH has issued regulations on the functioning of self-operating hospitals, emphasizing the importance of establishing a dedicated department that is responsible for developing health policies and procedures to govern the operation of these hospitals (Ministry of Health, 2010). Establishment of these autonomous clusters has increased the need for administrative policies and procedures that are anchored in Saudi Arabia's statutory mandates regarding the provision and delivery of health care, which conform to the guidelines of international accreditation bodies on patient safety and quality of health care delivery. For example, international health care quality accrediting bodies, such as the Joint Commission International, stress the importance of all health care professionals understanding an organization's health policy and procedures; these guidelines also require that all medical students and trainees comply with these policies and procedures (Joint Commission International, 2013).

Based on the above aspects, future clinicians will need additional knowledge and skills, apart from clinical competences, to work effectively in the newly created autonomous clusters. Such additional knowledge and skills form an important segment for health policy development. The findings of the present study hypothesize that there is little understanding of health policy principles among health care professionals in Saudi Arabia and feel that this situation has created a gap in the processes of introduction, formulation, and implementation of health care policies in some self-operating health care facilities. To address this problem, eight seminars and workshops were conducted among health professionals working at senior management level in four regional locations throughout Saudi Arabia. The aim of these workshops was to assess their basic knowledge about health policy as well as to facilitate their understanding about health policy development with context to Saudi Arabia and its analysis. Participants received continuing medical education credits for attending the workshops.

These workshops constituted the first-ever activity conducted to develop a health policy training course to be used as foundational material for health policy education in the context of Saudi Arabia. To the author's knowledge, the present study is the first to compare health policy knowledge among health care professionals in order to assess the needs for teaching health policy to medical professionals.

MATERIAL AND METHODS

Quantitative and qualitative methods were used to achieve the study objectives. Quantitative methods were used to compare the knowledge of health policy among health care professionals. Study participants were recruited in four cities (Riyadh, Jeddah, Dammam, and Al-Hasa) of Saudi Arabia. The workshop was conducted

in eight sessions at the four study sites. All workshops at each facility were of two days, conducted from October 2018 to February 2019, the workshops were conducted in Riyadh four times whereas it was conducted one time in other cities. The details of the participants have shown in Table 1.The number of participants varied among workshops but these were planned so as not to exceed 48 attendees (for a maximum of six teams with no more than eight people per team). Participants included 104 consultant physicians (chairs or deputy chairs), 99 management professionals, and 82 professionals from other services departments.

Medical service professionals included consultant physicians providing medical services in different departments (internal medicine; surgery; emergency department; oncology; cardiology; pediatric medicine; family medicine; ear, nose, and throat; labor and delivery; anesthesia; and pathology). Management group participants worked in administrative departments (finance and patient care services). Participants working in other services included those in nursing, pharmacy, and other specialties from non-clinical services.

A one-page survey questionnaire in English was distributed to participants before and after each workshop. During registration, participants were given a written pre-test questionnaire by the workshop assistants; completed questionnaires were returned prior to entry to the workshop venue. The pre-test survey collected information on department/area (medical, management, or other services) and position title of each participant; the names of participants were not recorded. Workshop site and regional information were previously recorded on the forms. Workshop participants were verbally informed that the information they provided would remain confidential. On the questionnaire, participants were required to match a list of terms (column A) with their definitions (column B). The questionnaire was designed in such a way as to ensure that all topics covered in the workshop were addressed.

The survey was meant to gauge awareness of and familiarity with terms and differences among health care professionals. Participants completed the same survey after the end of the workshop, with the addition of one open-ended question asking respondents to provide feedback on the workshop. Workshop assistants then collected post-test questionnaires and submitted them to the organizing team. The post-workshop questionnaire was designed to cover the main learning outcomes. Because there is no agreed content for the health policy training curriculum (Patel et al., 2011; Heiman et al., 2015; Kidd et al., 2016) the questionnaire and content of the workshop were reviewed by three experts in health policy, medical education, and health systems.

A pilot test was conducted among 10 people with different health care backgrounds, to assess their understanding of the questions and seek their advice about the questions. The results of this pilot test allowed the organizing team to assess the questions and to reconsider some of the possible answers. It was at this stage that, to minimize guessing of correct answers, more entries were included in column B. Appendix 1 presents the main questions, with their correct answers. In addition, some respondents commented that the questions were long and that those in senior positions would not like it to be known that they did not know the answers to the questions. Based on these comments, items querying information that could lead to personal identification of individual participants were minimized, but the main matching questions were not changed.

The workshops were designed to be intensive, covering all course material in 2 days. Workshops followed an interactive format, allowing for the exchange of ideas between presenters and participants. Information on topics associated with Saudi health care policy was adapted from materials that have been published elsewhere (Al Khamis, 2016). Participants from similar professional areas were grouped together and tasked with developing a micro policy specific to their assigned areas. The output was then presented and used as a springboard for a discussion of different topics and challenges on policy development. Although most participants were Saudi nationals, the workshop was conducted in English, including workshop materials, group discussions, and group presentations. Survey results before and after the workshop were assessed using Microsoft Excel 2013.

Qualitative semi-structured interviews were used to seek medical college deans' advice regarding the results of the study and assess their attitudes with respect to teaching health policy to medical professionals; interviews were conducted from July 2019 to January 2020. A qualitative approach was followed owing to its flexibility and allowing for in-depth understanding of participants' attitudes; this approach can help to identify gaps in health policy education that cannot otherwise be identified using survey-based research methods (Berg, 2011; Mullen & Reynolds, 1978). Eight deans of medical colleges with a minimum 10 years' experience in academic and health care fields were interviewed.

The deans were all men with an academic title of at least assistant professor and were located in different cities (four from Riyadh, one from Medina, one from Al Majmaah, and two from Jeddah). Two deans were consultant physicians with the MOH Vision Realization Office, which gave them the advantage of a deeper understanding of the MOH transformation. To provide a good basis for discussion, participates were asked about their understanding of the meaning of health policy and what issues are covered by health policy. All deans were briefed about the workshop outcomes and were asked three main questions, as follows: (1) How do you evaluate physicians' health policy knowledge based on the workshop outcomes? (2) Do you think it is important to teach health policy to physicians? If the answer is yes, at what level along the career path should this be taught?

RESULTS AND DISCUSSION

The 285 workshops participants were senior management level professionals (director, chair/deputy chair, or higher) with three different backgrounds: medical services, management, or other services (clinical and non-clinical services). The main workshop content, teaching methods, and learning outcomes are illustrated in Table 1.

Table 1. Health policy workshop timetable, contents, and learning outcomes

	Session	Teaching and learning methods	Learning outcomes	References
Day 1: Policy, policy categories, and policy analysis	What is health policy?	Interactive presentation Individual Exercise: What is the policy from your perceptive?	The participants will be able to understand variations in policy terms and usage (general policy / health policy / public policy)	[22, 23]
	Health policy categories	Interactive presentation	The participants will be able to understand the difference between micro and macro policies	[10]
	Micro health policy categories and the differences between policy and procedure	Interactive presentation •Individual exercise and group discussion / presentation: Determining the classification of example policies as micro or macro policy •Determining the classification of example as Administrative Policy and Procedure (APP), Clinical Practice Guidelines (CPG), or departmental policy and procedure (DPP).	The participants will be able to understand the differences between APP, CPG, and DPP	[10, 24, 25]
	Developing micro policy	Interactive presentation	The participants will be able to understand the following: •how to start developing a micro policy •the main components of the analytical process •plan, policy, procedure, and relationship •the main templates used in writing APP, DPP, and CPG	[10, 23, 25, 26]
	Macro policy	Interactive presentation	The participants will be able to understand the following: •the difference between global policy and national policy •healthcare system components •type of health care financing systems •the relationship between national and global policy	[27-31]
	Health policy analysis	Interactive presentation •Group work: The participants are grouped		[9, 23, 32-34]

Continue Table 1

		according to their specialties and develop a policy (micro or macro) using the analysis steps	The participants will be able to understand the following: •the definition of health policy analysis •health policy development stage in theory •health policy triangle in theory •some of the health policy analysis tools •the use of a sample tool for policy makers •the health policy analysis checklist •the complexity of the policy process in the real life •the main challenges facing policy developments in different stages	
Day 2: The actors in the policy process and policy context; main challenges in implementing health policy; user training on policy writing	Power and types of actors	Interactive presentation	The participants will be able to do the following: •Identify different kinds of power •Understand the main actors in the policy process •Identify the main players in the policy process •Illustrate the influence of actors on the policy process •Analyze the relationship between policy content and context	[9, 32]
	Policy context and content; Main policy implementation challenges	Interactive presentation Group Discussion: Based on the policy you developed with your team yesterday, please define the main challenges facing the implementation of your policy	The participants will be able to do the following: •Understand the main contextual factors influencing policy development •Analyze the main challenges facing the policy implementation process	[32, 35, 36]
	Writing health policy	Interactive presentation	The participants will be able to do the following: •Identify the main tips in writing policy and procedure •Understand the main policy format writing •Understand how to write a professional policy and procedure	[26, 37]

Table 2 illustrates participants' characteristics. More than 34% of participants were in the management group, and more than 36% were consultant physicians. The number of participants in each workshop ranged from 30 to 45. The highest proportion of participants with medical backgrounds was in Jeddah (Workshop 6, 16/40 participants) whereas the lowest proportions were in Dammam (Workshop 3, 10/34 participants) and Riyadh (Workshop 8, 10/31 participants). Across the four facilities, 86% (246/285) of participants completed both the pre- and post-workshop questionnaires. When only one pre-/or post-workshop questionnaire was submitted by a participant, the data were considered incomplete and excluded from the analysis.

Table 2. Main characteristics of participants

Facilities / Group	Medical			Other Services			Management		
	# of Participants	Pre-Workshop Average Score	Post--Workshop average Score	#of Participants	Pre-Workshop average Score	Post--Workshop average Score	# of Participants	Pre-Workshop average Score	Post--Workshop average Score
Workshop 1 (Riyadh)	11*	3	8	5	5	8	13*	7	10
Workshop 2 (Jeddah)	13*	2	8	12*	4	8	12*	5	8
Workshop 3 (Dammam)	10	2	7	10*	3	7	11*	4	8
Workshop 4 (Al Hasa)	12*	3	8	7*	3	7	13*	5	8
Workshop 5 (Riyadh)	13*	3	9	3*	5	8	11	6	9
Workshop 6 (Jeddah)	15*	2	8	9*	6	10	9*	7	10
Workshop 7 (Riyadh)	13	4	10	9*	5	8	8*	6	10
Workshop 8 (Riyadh)	9*	3	10	11*	4	10	7*	6	10
Total Participants per Group/ Mean Scores Per Group	96	2.75	8.50	66	4.38	8.25	84	5.75	9.13

*39 questionnaires were missing

Table 3 shows that senior consultant physicians had the lowest pre-workshop scores (mean 2.75 points) whereas the other services and management groups had mean scores of 4.38 and 5.75, respectively. However, after the workshop, the difference in knowledge scores was minimal across the three groups, with the medical, other services, and management groups scoring a mean 8.5, 8.25, and 9.13 points, respectively. Among participants with a medical background, the lowest pre-workshop scores were observed in Workshop 2 (Jeddah), Workshop 3 (Riyadh), and Workshop 6 (Riyadh), with a mean 2 of 10 possible points. The highest mean scores following the workshop among participants with a medical background were in Workshops 7 and 8 (both in Riyadh).

Most participants did not thoroughly understand the relationship among global health policy, national health policy, and administrative health policy within their facilities. In addition, there were clear misunderstandings of the stages of the public policy process. The study was unable to conduct a detailed analysis of the effects of participants' individual characteristics because only limited data were available. This was because one of the outcomes of the pilot study was to minimize descriptive variables collected together with survey questions, to encourage greater participation and ensure the confidentiality of participants. However, the semi-structured interviews conducted with deans of medical colleges enabled analysis of the views and attitudes among these senior health professionals about teaching health policy at some point during physicians' careers. The qualitative aspect of this assessment enabled clarification of attitudes toward teaching health policy for medical professionals. For example, participants tended to see policy as rigid statements that cannot be modified or changed, despite changes in the environment or circumstances.

There was agreement in the semi-structured interviews among medical college deans about the importance of teaching the basic principles of health policy as part of the undergraduate medical college curriculum. During and after the workshop, participants realized the importance of the training for their practice as clinicians and medical educators. There was also agreement in the semi-structured interviews among medical college deans regarding the importance of health policy education at some point in a physician's career path, but they expressed different approaches on how health policy knowledge should be enhanced. For example, some deans believed that health policy could be included as an elective course within undergraduate programs whereas others felt it should be part of a health care systems course; yet other deans expressed that the curriculum should include one core course containing the different principles of leadership, health systems, and health policy.

One dean stated, "Previously, the medical curriculum was focused on diseases and sciences in general terms, but the new medical college curriculum has been shifted globally to include soft skills such as communication, health systems, health policy, and research". The deans referred to the national outcomes/competency framework for Saudi medical education and practice. This framework declares the national health systems including organizations, policies, and procedures as a part of medical students' curriculum (The Saudi Dean`s Committee, 2017), there are variations in implementation of the national framework among medical colleges.

All deans agreed about the importance of teaching health policy at postgraduate level, but that the scope and length of instruction might differ according to specialty, depending on the level of exposure to the community in a particular specialty. For example, teaching health policy is important in community medicine and emergency medicine whereas it might be less so in surgical specialties. One dean stated, "We have just finished writing the main competencies for family medicine for the Saudi Commission for Health Specialties and one of the main competencies is to be a manager and leader and to be able to accommodate oneself within the Saudi health system, including the cost effectiveness of the health system". This move toward teaching health policy and health systems is supported by the program outcomes of the Canadian framework, CanMEDS (The Royal College of Physicians and Surgeons of Canada, 2019).

Table 3. Mean scores of participants by specialty and facility

	Medical		Other services		Management	
Workshop/Facility	Pre-workshop	Post-workshop	Pre-workshop	Post-workshop	Pre-workshop	Post-workshop
Workshop 1 (Riyadh)	3	8	5	8	7	10
Workshop 2 (Jeddah)	2	8	4	8	5	8
Workshop 3 (Dammam)	2	7	3	7	4	8
Workshop 4 (Al-Hasa)	3	8	3	7	5	8
Workshop 5 (Riyadh)	3	9	5	8	6	9
Workshop 6 (Jeddah)	2	8	6	10	7	10
Workshop 7 (Riyadh)	4	10	5	8	6	10
Workshop 8 (Riyadh)	3	10	4	10	6	10
Mean	2.75	8.5	4.38	8.25	5.75	9.13

Regarding teaching health policy after postgraduate education as a part of professional development, all deans agreed that this might differ based on the role of consultant physicians. For example, if physicians are assigned a managerial role in health care, they must have extensive knowledge and skills in managerial and health systems, including health policy principles. Otherwise, these professionals do not require advanced knowledge in health systems and health policy principles. All deans felt optimistic that health policy knowledge in Saudi Arabia is gradually increasing. Changes in the health system have forced physicians to practice differently. Previously, there was little accountability regarding services provided to patients. Physicians' salaries were unrelated to the degree of effort and level of practice. With the current health care transformation, physician's awareness and practice will undoubtedly change. One of the interviewed deans expressed, "Our teaching is hospital oriented and not health oriented. Health transformation programs have changed physicians' attitudes toward the leading health systems and health policy. Today, population health management, value-based care, and so on have become part of physicians' concerns and discussions".

The workshops described here were conducted at different hospitals in four cities of Saudi Arabia. These represent the first intervention conducted in the Saudi Arabian context to target health care professionals and address some of the basic principles of health policy. The aim of these workshops was to identify and assess knowledge among health care professionals regarding basic health policy principles. Workshop participants included medical, management, and other health care professionals from different health facilities in Saudi Arabia. This is the first study evaluating health policy knowledge among different health care professionals and the first report of heath policy knowledge in Saudi Arabia.

Mean scores of the initial assessment revealed that health policy knowledge among senior medical professionals was lower than that of senior staff working in management and other services. However, after the workshop, scores were improved among all medical staff members. The lower levels of health policy knowledge among medical professionals before the training can be attributed to this group having received no exposure to health policy topics during their formal education, as has been reported previously,(Greysen et al., 2009). Some prior studies have indicated that health policy should form part of physicians' medical education, (Clancy et al., 1995; Goetz et al., 2010; Sabat et al., 2020); however, there is limited evidence on how to develop and evaluate health policy curricula. Although several curricula have been proposed (Chinitz, 2002; Greysen et al., 2009; Heiman et al., 2015; Kidd et al., 2016), there is no consensus as to which content should be included in health policy training (Patel et al., 2011; Khatana 2017; Kiendrebeogo, Allegri and Meessen, 2020).

.As part of reforming the health care sector, the Saudi government will move to extend the number of health insurance beneficiaries (The Council of Economic and Development Affairs, 2016). Following the health care reforms, health professionals will increasingly be required to have knowledge regarding not only clinical

practice but also health policy, (Kidd et al., 2016). In Saudi Arabia, formulation of a national health policy is necessary to align with the MOH's strategic plan and Saudi Vision 2030. Academics must actively participate in this development by assisting health care professionals to understand the basic concepts of health policy in the context of Saudi Arabia's unique environmental, demographic, and political system. Although there is agreement about providing health policy education during undergraduate and postgraduate medical training as well as professional development, there is no mechanism to ensure that all medical schools are aligned in this regard and moving in the same direction.

The participants in this study expressed agreement that the principles and concepts taught in the workshop were important and beneficial to health care professionals. However, prior to the workshop, some medical staff members expressed reservations about attending because they believed that the workshop would not be relevant to their professional activities. This initial attitude, which was also observed in a prior study(Greysen et al., 2009), highlights the importance of communicating with medical professionals to increase their awareness of the benefits this kind of training can provide in their work. In this study, all venues and facilities were provided by the hospitals, and use of assistants helped to increase efficiency of the activities conducted during the workshops. For example, assistants were assigned to each team to guide participants in understanding the assignments and completing the workshop exercises.

The workshops described in this article covered the main health policy issues relevant to health care providers and used different approaches such as lectures, discussions, and group projects. However, there is a need to develop a national health policy program that elaborates on the other health policy domains, particularly with movement of the Saudi government toward reforming the national health system (The Council of Economic and Development Affairs, 2016). Thus, developing a national health policy curriculum should be a priority, with participation by the academic community, to assist medical and public health programs in providing training for health care professionals that will serve to increase their knowledge of health policy.

This study has some limitations. The study was unable to conduct a detailed analysis of the effects of participants' individual characteristics because only limited data were available; this was because one of the outcomes of the pilot study was to minimize descriptive variables collected together with survey questions, to encourage greater participation and ensure the confidentiality of participants. However, the semi-structured interviews conducted with deans of medical colleges enabled analysis of the views and attitudes among these senior health professionals about teaching health policy at some point during physicians' careers. The qualitative aspect of this assessment enabled clarification of attitudes toward teaching health policy for medical professionals.

CONCLUSION

The findings of the current study revealed that medical professionals had lower levels of knowledge regarding health policy than those in other specialties because they had received no health policy training during their medical education. Introduction of the health policy workshop described in this article helped to reduce the knowledge gap among health care professionals in this regard. Although the intervention in this study presented some health policy content that can be used in teaching health policy, there is still a need to formulate a national health policy program that is aligned with the current government health care reforms. The present study results stress the importance of communicating with medical staff members and increasing awareness of the benefits of health policy training for their work, to minimize resistance by senior medical professionals to participating in health policy workshops.

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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Antibacterial, Antioxidant and Anti – Cancerous Activities of *Adiandra megaphylla* Hu Leaf Extracts

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ABSTRACT

Adinandra megaphylla Hu which belongs to *Adinandra genus*, Theaceae family, only narrowly distributed in Vietnam. Biological activities of this plant's secondary compounds have been left open yet. In this study, the antibacterial, antioxidant abilities and inhibiting cancer cell lines activity of leaf extract of *A. megaphylla* collected in Lao Cai province, Vietnam were initially investigated. Poultice extracted from leaves of *Adinandra megaphylla* with three solvents of ethanol, ethyl acetate and dichloromethane were quantified and determined composition of the polyphenol, flavonoid and coumarin groups. The ethanol extract, ethyl acetate extract and dichloromethane extract have been shown to inhibit the growth of *Staphylococcus aureus, Bacillus subtilis, Seratia marcessens, Sarcina lutea, Lactobacillus plantarum* and *Escherichia coli* at concentration of 200 µg mL^{-1}. The ethyl acetate extract and dichloromethane extract have DPPH free-radical activities; the EC50 value reached 30.3 and 33.2 µg mL^{-1}, respectively. In particular, antimicrobial and free-radical activities of the dichloromethane extract were better than ethanol and ethyl acetate extracts. Extracts from *A. megaphylla* showed inhibition of gastric, lung and breast cancer cell lines with values of 67.76, 77.02 and 84.46 µg mL^{-1}, respectively. Research results show that *A. megaphylla* is a potential plant containing many compounds with antibacterial, antioxidant abilities and inhibiting cancer cell lines.

KEY WORDS: *ADINANDRA MEGAPHYLLA*, ANTIOXIDANT, ANTIBACTERIAL, ANTI-CANCER, POULTICE.

INTRODUCTION

Vietnam has high potential for medicinal plants, which their chemical composition and pharmacological activities of some herbaceous species have been studied in previous studies (Hung et al., 2019; Minh et al., 2010; Vu et al., 2019), but there are still many species that have not been assessed their medicinal value, including *Adinandra megaphylla* of the genus *Adinandra*, the tea family (Theaceae). In the world, there are about 85 species in the *Adinandra* genus distributed in Bangladesh, Cambodia, China, India, Indonesia, Southern Japan, Laos, Malaysia, Myanmar, New Guinea, Philippines, Sri Lanka, Thailand, Vietnam and African rainforests. In China, the genus *Adinandra* has 22 species, of which up to 17 are endemic (Min and Bruce, 2007). In "An illustrated flora of Vietnam", Pham Hoang Ho (1999) indicated that the genus *Adinandra* in Vietnam contains about 11 species which scattered throughout the country.

Species of the genus *Adinandra* consist of secondary compounds with antibacterial, anti-inflammatory, antioxidant, anti-free radical and anti-cancer activities. However, studies on the genus *Adinandra* have been

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mainly focused on the species *Adinandra nitida*. In 2008, Liu et al. isolated camellianin A from *A. nitida* Merr. ex Li by column chromatography and determined its content by HPLC. At the same time, their study demonstrated high antioxidant capacity from flavonoids extracted by DPPH and free radical cleaning method (Liu et al., 2008). In addition, Liu et al. (2013) optimized flavonoid extraction method and obtained camellianin A from *A. nitida* leaves; flavonoids were also reported to have antioxidant ability at the concentration of 0.02 mg mL^{-1} (Liu et al., 2013).

Thus, previous studies have shown that flavonoids like epicatechin, apigenin, quercitrin, camellianin A and camellianin B to have biological and antioxidant activities. Chemical composition of *A. nitida* has been isolated and determined by Wang et al. (2008) using column chromatography. The structure of saponins compounds comprise 6 types, including 2alpha, 3alpha, 19alpha- trihydroxy-olean-12-en-28-oic acid-28-O-beta-D-glucopyranoside; arjunetin; sericoside; glucosyl tormentate; nigaichigoside F1 and arjunglucoside I. Among of which, 2alpha, 3alpha, 19alpha-trihydroxy-olean-12-en-28-oic acid-28-O-beta-D-glucopyranoside is a new substance. The remaining substances were first discovered in *A. nitida* (Wang et al., 2008).

Moreover, in the *Adinandra* genus, *Adinandra lienii* was initially studied for its geographical distribution and *matK* sequence to help identify this species in Lao Cai province, Vietnam. Meanwhile, there have been not any studies on chemical composition and biological activity of total extracts from *Adinandra megaphylla* yet. In this study, we present results of qualitative analysis of chemical composition and evaluation of antibacterial, antioxidant, anti-cancer activities of the *A. megaphylla* Hu extracts.

MATERIAL AND METHODS

Figure 1: Morphological characteristics of *A. megaphylla* Hu collected in Lao Cai province, Vietnam. Life form (A), branches with buds (B, C), flowers (D)

***A. megaphylla* samples:** *A. megaphylla* samples was collected from Lao Cai province, Vietnam in the 1200-1800 m altitude at 21°59'15''N; 104°19'28''E. *A. megaphylla* Hu samples (branches with leaves and flowers) were collected to determine the scientific name in laboratory. *A. megaphylla* Hu leaves were used for poultice extraction with ethanol, ethyl acetate and dichloromethane. The scientific name of species is determined by comparative morphological methods according to monograph including "An Illustrate Flora of Vietnam" and "Flora of China" (Figure 1).

Bacterial strains and cancer cell lines: Bacterial strains (*Bacillus subtilis, Serratia marcescens*, *Escherichia coli*, *Sarcina lutea*, and *Lactobacillus plantanum*) were selected for the antibacterial activity assay. They were grown in liquid Luria-Bertani (LB) medium (0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 1.0% (w/v) NaCl, pH 7.0) overnight at 28°C, and the diluted bacterial suspension (106ˆmL^{-1}) was ready for detection. Solid LB medium contained additionally 2.0% (w/v) agar. Cancer cell lines, including the breast cancer cell line (MDA-MB-231), the stomach cancer cell line (AGS) and the lung cancer cell line (A549) were used in cytotoxicity assays.

Materials and chemicals: Yeast extract and peptone were purchased from Bio Basic Inc. (USA); Ethanol, ethyl acetate and dichloromethane were from Fluka (China); TLC silica gel 60 F254 was from Merck (Germany).

Method of sample preparation: The leaves of *A. megaphylla* are washed thoroughly, then cut into pieces and dried at a temperature of 50°C to constant mass. The crushed sample is extracted twice with ethanol in an ultrasonic machine at room temperature. The crude extracts were collected by solvent removal under reduced pressure conditions, at 50°C and extracted with solvents of dichloromethane and ethyl acetate. The residue of ethanol, dichloromethane and ethyl acetate were cleaned solvent and dried at 50°C to collect ethanol extract, dichloromethane extract and ethyl acetate extract, respectively.

Polyphenols were detected by reaction with iron salts (III)/sulfuric acid: Reaction with iron salts (III): 5 mL of ethanol extract were added into two tubes, denoted by I and II, respectively. The tube II was supplemented with 0.5 mL iron salts (III), shake and observe color. Depending on the number and location of hydroxyl groups in polyphenol molecules, results are green, blue or brown.

Reaction with sulfuric acid: 2 mL of ethanol extract were added into two tubes, denoted by I and II, respectively. The tube II was supplemented with 1-2 drops of H_2SO_4, shake and observe color. Add H_2SO_4 concentrate to flavones and flavonols to give a deep yellow; to chalcones and aurones to produce a red, crimson red or bright red solution; to flavanones to give orange red.

Flavonoids were detected by reaction with hydrochloric acid and magnesium powder:The tube contained 0.05g ethanol extract and 10 mL CH_3OH, which were shaken, heated to dissolve and filtered through filter paper. 2 mL of filtrate was added into two tubes, denoted by I and II, then was added a pinch of magnesium powder and shaken. The tube II was supplemented with five drops of HCl and boiled for 3 mins. The solution changed to yellow, red to green colors, as it contained flavonoids. Coumarin was detected by reaction with NaOH solution:

2 mL of ethanol extract were added into two tubes, denoted by I and II. The tube II was supplemented with 0.5 mL of 10% NaOH solution. Two tubes were boiled, cooled down to room temperature and added 4 mL distilled water. The tube II is more transparent or clear than tube I, indicates the presence of coumarin. When the two test tubes were added with a few drops of HCl, the solution turned a dull yellow, so the coumarin was determined following this method (Nguyen and Hung, 2008).

Thin layer chromatographic method (TLC): Ethanol extract from *A. megaphylla* leaves were detected by TLC (3.5 × 10 cm layer of silica gel 60), performed with two mobile phases of n-hecxane/acetone (1:1, v/v) and dichlomethane/n-hexane (3:1, v/v). The products were visualized by spraying the TLC plate with 10% (v/v) sulfuric acid in ethanol and incubating at 100°C until color appeared.

Determination of antibacterial activity of extracts: Antibacterial activity of extracts was performed according to the method of Mahesh and Satish (2008). In order to determine antibacterial activity of the extracts, 70 mL of diluted bacterial suspension (106^mL^{-1}) was brushed on 0.5-cm-thick LB plates. The LB plates were perforated with 0.5-cm-diameter holes, and each hole was supplemented with 100 mL of each ethanol, ethyl acetate and dichloromethane extract from *A. megaphylla* Hu leaves with different concentrations (20, 60 and 200 µg mL^{-1}) or with DMSO for the control. The inhibition activity of extracts against bacterial growth was observed after incubation at 30°C for 18-40 hours. The antibacterial levels were determined by diameter of inhibition zones (in millimeters) around the holes. The diameter of antibacterial ring was determined by the formula: H = D-d (mm). In which: D is the diameter of the antibacterial ring from the center of perforations (mm); d is the diameter of perforated agar (mm).

Determination of oxidation activity of extracts: Antioxidant activity of *A. megaphylla* extracts was determined by Tabart et al. (2009) using DPPH radical scavenging. 100 µL of each extract at five concentrations, including 0.5, 2, 8, 32 and 64 µg mL^{-1} were added with 2.9 mL of 0.1 mM DPPH solution mixed in methanol solution, shook and left in the dark for 30 min at room temperature, the absorbance was measured at 517 nm. The inhibition of DPPH radical by the samples was calculated by following formula: DPPH activity (%) = 100 × (Ac - As)/Ac, in which Ac: the absorbance of control, As: the absorbance of sample. Antioxidant activity was determined based on EC_{50} values (the concentration of DPPH free radical scavenging samples is 50%) (Tabart et al., 2009).

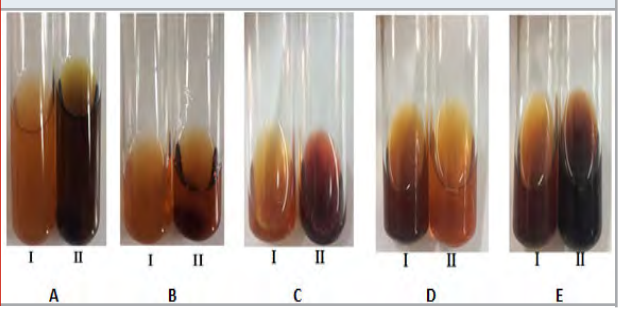
Cytotoxic assays: Cancer cytotoxicity was determined using the method of Monks et al. (1991) (Monks et al., 1991). Poultice extracted from *A. megaphylla* leaves was prepared and tested at four concentrations, including 100, 20, 4 and 0.8 µg mL^{-1}. Ellipticine was used as a reference at four concentrations: 10, 2, 0.4 and 0.08 µg mL^{-1}. Dimethyl sulfoxide (DMSO) at 10% concentration was used as a negative control. Total protein content of a cell is determined based on the optical density (OD) when proteins of the cell are stained with sulforhodamine B (SRB). The OD results were read on a wave step of 515 nm in an ELISA Plate Reader. OD values are proportional to the amount of SRB, which is attached to protein molecules. The larger the OD value, higher the amount of protein and higher the amount of cells. Cytotoxicity was expressed as the concentration of drug that inhibited cell growth by 50%. Inhibitory concentration 50% (IC_{50}) is the concentration of the sample at which it can inhibit 50% of cells. The substance is considered to have good activity when IC_{50} = 5 mM (Hughes et al., 2011).

RESULTS AND DISCUSSION

Results of thin layer chromatographic analysis show that, there were 7 marks in the ethanol extract and 6 marks in the ethyl acetate extract for the n-hexane/acetone at a 1:1 ratio (Figure 2A). There were 3 marks in the ethanol extract and 6 marks in the ethyl acetate extract for the dichloromethane/n-hexane solvent system at a 3:1 ratio (Figure 2B). Thus, on the thin layer chromatographic analysis with two different solvent systems showed that the extract from A. megaphylla Hu has many bands with different colors.

Figure 2: Results of thin layer chromatographic analysis of ethanol extract (A) and ethyl acetate extract (B) in the n-hexane/acetone at a 1:1 ratio (I) and dichloromethane/n-hexane solvent system at a 3:1 ratio (II)

Figure 3: Color reactions for detection of polyphenols (A, B), flavonoids (C) and coumarin (D, E) in the ethanol extract from leaves of *A. megaphylla*. The ethanol extract before (I) and after (II) reacting with iron salts (III) (3A); with sulfuric acid (3B); with Mg in HCl solution (3C); with NaOH (3D); with HCl solution (3E).



The extract of *A. megaphylla* was quantified polyphenols, flavonoids and coumarin by different reagents. The results were shown in Figure 3.

Polyphenols in the ethanol extract was detected by reacting with iron salts (III), the solution in the tube II turned dark green due to reaction between polyphenols and iron salts (III). The solution in the tube II turned dark yellow (Figure 3B), if flavonoid reacted with sulfuric acid. According to flavonoids qualitative, the solution in the tube II changed color from yellow to dark red (Figure 3C). Coumarin was detected by reaction with 10% NaOH solution, the results showed in tube II is more transparent than tube I (Figure 3D). Then, when a few drops of HCl were added to both tubes, the tube II changed from dark opaque yellow to light transparent yellow color (Figure 3E). Therefore, it is clear from these results that the extract of *A. megaphylla* leaves contained polyphenols, flavonoids and coumarin. Previous studies have also demonstrated that leaves of *Adinadra nitida* contained total flavonoids Gao et al., 2010; Chen et al., 2017. Bioactive compounds in these plants have an important role in producing medicinal products as well as a basis for further research.

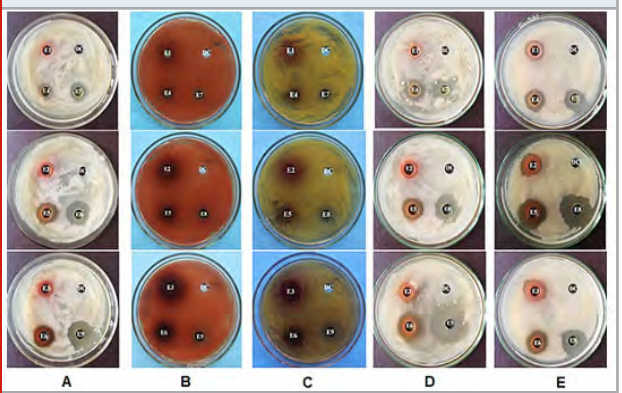
Antibacterial activity of ethanol, ethyl acetate and dichloromethane extracts from leaves of *A. megaphylla* Hu was tested at different concentrations using bacteria via the agar diffusion method (Table 1 and figure 4). The ethanol extract had no bactericidal effects at a concentration of 20 µg mL^{-1}, and had low activity at 60 and 200 µg mL^{-1} concentrations on *B. subtilis*. Whereas, the ethyl acetate and dichloromethane extract showed antibacterial activity against *B. subtilis* at all concentrations tested, and the dichloromethane extract at 200 µg mL^{-1} had the strongest antibacterial activity (Figure 4A).

Table 1. Antibacterial activities of extracts from leaves of *A. megaphylla* Hu

No.	Bacterial	Experimental concentrations of the extracts								
		E1	E2	E3	E4	E5	E6	E7	E8	E9
1	*B. subtilis*	-	+	+	+	++	++	++	+++	+++
2	*S. macescens*	-	-	-	-	-	-	-	-	+
3	*S. lutea*	-	-	-	-	-	-	-	++	+++
4	*L. plantarum*	+	++	++	++	++	+	+++	+++	+++
5	*E. coli*	+	+++	+++	++	+++	++	++	+++	+++

Note: (-) No inhibition (no antibacterial zones); (+) Weak inhibition (the diameters of inhibition zones are from 1 to 5 mm); (++) Inhibition (the diameters of inhibition zones are from 6-10 mm); (+++) Strong inhibition (the diameters of inhibition zones are >10mm).
The ethanol extract at concentration of 20 (E1); 60 (E2) and 200 (E3) µg mL^{-1}; the ethyl acetate extract at concentration of 20 (E4); 60 (E5) and 200 (E6) µg mL^{-1}; the dichloromethane extract at concentration of 20 (E7); 60 (E8) and 200 (E9) µg mL^{-1}.

Figure 4: Antibacterial activities against *B. subtilis* (A), *S. marcescens* (B), *S. lutea* (C), *L. plantarum* (D) and *E. coli* (E) of *A. megaphylla* Hu extracts; DC: Dimethyl sulfoxide (DMSO) served as a control.



The ethanol, ethyl acetate and dichloromethane extracts had no antibacterial activity against *S. macescens* at all concentrations (Figure 4B). For *S. lutea* bacteria, the ethanol extract, the ethyl acetate extracts at concentration of 20, 60 and 200 µg mL^{-1} and the dichloromethane extract at concentration of 20 µg mL^{-1} had no antibacterial activity. While, the dichloromethane extract at 60 and 200 µg mL^{-1} concentrations had strong antibacterial activity against *S. lutea* (Figure 4C). The ethyl acetate and dichloromethane extract had antibacterial activity against *L. plantarum* at all concentrations tested, and the strongest antibacterial activity was observed with the dichloromethane extract at 200 µg mL^{-1} (Figure 4D). The ethanol, ethyl acetate and dichloromethane extracts had antibacterial activity against *E. coli* at all concentrations; The strongest resistance activity was at 60 µg mL^{-1} for the dichloromethane extract, followed by the ethyl acetate (Figure 4E).

Antibacterial activities of extracts from leaves of *A. megaphylla* Hu on five bacterial strains showed that: (1) The ethanol and ethyl acetate extracts were able to inhibit *B. subtilis, L. plantarum, E. coli*, and no inhibit *S. lutea, S. macescens*. (2) The dichloromethane extract had inhibited *B. subtilis, S. marcessens, S. lutea, L. plantarum*, *E. coli*. (3) Antimicrobial activities of dichloromethane extract is better than the ethanol and ethyl acetate extracts.

Table 2. Antioxidant activities of extracts from leaves of *A. megaphylla*

Concentrations (µg mL^{-1})	DPPH free radical scavenging activity (%)		
	Ethanol extract	Dichloromethane extract	Ethyl acetate extract
0.5	0	0	25.4 ± 0.54
2.0	0	0	28.6 ± 2.02
8.0	0	0	29.2 ± 4.04
32	48.2 ± 4.45	0	52.7 ± 5.52
128	71.8 ± 5.28	40.5 ± 0.35	75.7 ± 0.96
EC50	33.2 ± 0.42	> 128 ± 0.98	30.3 ± 3.26

Antioxidant activities of extracts from leaves of *A. megaphylla* Hu: The results showed that the DPPH free radical removal efficiency of the ethanol, dichloromethane and ethyl acetate extracts were directly proportional to the extract concentrations. The free radical removal efficiency increased from 0 to 75.7% with the increase of extract concentration from 0.5 to 128 µg mL^{-1}. The ethyl acetate extract proved to have the strongest DPPH free radical activity with an EC50 value of 30.3 µg mL^{-1}. The ethanol extract showed DPPH free radical activity with an EC_{50} value of 33.2 µg mL^{-1}. By contrast, the DPPH free radical activity of dichloromethane extract was very weak with the EC_{50} value >128 µg mL^{-1} (Table 2).

Cytotoxic activities of the ethanol extract from leaves of *A. megaphylla* Hu against cancer cell lines: According to Gao et al. (2010), camellianin A, a flavonoid from leaves of *Adinandra nitida*, was determined to inhibit proliferation and apoptosis of liver cancer cells (Hep G2) and breast cancer (MCF-7) (Gao et al., 2010). Some heterocyclic compounds containing coumarin-related properties such as anti-inflammatory (El-Haggar and Al-Wabli, 2015), antibacterial (Shi and Zhou, 2011), antiviral (Tsay et al., 2014) and anti-cancer (Jacquot et al., 2007). Moreover, coumarin inhibited Hep2 cell growth and showed typical characteristics of apoptosis including the morphological changes and DNA fragmentation (Mirunalini et al., 2014).

In our study, the *A. megaphylla* extracts was found to contain flavonoids and coumarin compound, however it is necessary to identify whether or not they are resistant to cancer cells. Therefore, we preliminarily determined the anti-cancer activities of ethanol extract from leaves of *A. megaphylla* as a basis for efficient purification of flavonoid and coumarin compounds. The cytotoxic activity of ethanol extract against three cancer cell lines, including MDA-MB-231 (breast cancer cell line), AGS (stomach cancer cell line) and A549 (lung cancer cell line) was investigates in this study. The results showed that the extract of *A. megaphylla* has strong cytotoxic activity against MDA-MB-231, AGS and A549 with IC_{50} values are 84.46, 67.76 and 77.02 µg mL^{-1}, respectively (Table 3).

Table 3. Cytotoxicity of extract from leaves of *A. megaphylla* against human cancer cell lines in vitro (IC_{50}, µmol L^{-1})

Concentrations (µg mL^{-1})	Inhibition of human cancer cell line growth (%)		
	A549	AGS	MDA-MB-231
100	73.07 ± 0.66	84.61 ± 3.20	65.06 ± 0.41
20	5.96 ± 3.06	8.20 ± 2.62	4.64 ± 0.66
4	1.25 ± 1.59	3.44 ± 2.13	0.90 ± 0.08
0.8	-2.63 ± 0.80	-3.97 ± 0.29	-1.66 ± 0.74
IC50	77.02 ± 5.27	67.76 ± 3.31	84.46 ± 9.09

Note: IC_{50} values are means from three independent experiments (average ± SD) in which each compound concentration was tested in three replicate wells. Ellipticine (as a reference compound) was the positive control and assayed at concentrations of 10, 2, 0.4 and 0.08 µg mL^{-1}.

In particular, the inhibitory effect of the extract against stomach cancer cell line is highest, followed by lung cancer cell line and the lowest is the breast cancer cell line. Thus, the extract from *A. megaphylla* has activity against cancer cell lines, including the breast cancer cell line, the stomach cancer cell line and the lung cancer cell line. This result is the basis for the isolation of pure compounds with anti-cancer properties.

CONCLUSION

Results of thin layer chromatographic analysis using n-hexane/acetone at a 1:1 ratio and dichloromethane/n-hexane solvent system at a 3:1 ratio had been identified to contain polyphenols, coumarin in the extracts from leaves of *A. megaphylla* Hu. The ethanol and ethyl acetate extracts were able to inhibit *B. subtilis, L. plantarum*, *E. coli*, but not inhibit *S. lutea, S. macescens*. The dichloromethane extract had inhibited *B. subtilis, S. marcessens, S. lutea, L. plantarum, E. coli*. DPPH free radical activity of the dichloromethane extract is strongest in comparison to the ethanol and ethyl acetate extracts, the EC_{50} value of dichloromethane extract is 30.3 µg mL^{-1}. The ethanol extract from leaves of *A. megaphylla* Hu has activity against breast, stomach and lung cancer, the IC_{50} values reached to 84.46, 67.76 and 77.02 µg mL^{-1}, respectively. Thus, the dichloromethane

extract showed stronger biological activities comparing to the ethanol and ethyl acetate extracts. These research results have demonstrated that *A. megaphylla* contains bioactive and pharmacological compounds, which is a new potential source for isolating these compounds to produce medicine.

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Screening of Antimicrobial and Antioxidant Activities of *Moringa oleifera* Lam. Leaf Extracts Against Multidrug Resistant Pathogenic Bacteria

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ABSTRACT

Antibiotic resistance is a problem that continues to challenge the healthcare sector in a large part of the world. It is very important to control that problem, so the discovery of new active compounds and antibiotic has focused on screening bacteria for new growth inhibitory compounds. This study aimed to investigate the antibacterial, antioxidant potential and phytochemical composition of extracts of *Moringa oleifera* Lam. leaves in methanol, ethyl acetate and hexane against clinically resistant bacterial isolates. A total of 8 bacterial isolates were selected to analyze the antibacterial and antioxidant potential of various *Moringa oleifera* leaf extracts. Antimicrobial susceptibility test was performed by Kirby-Bauer disc diffusion technique and MIC and MBC values were also recorded. Antioxidant potential was determined based on the free radical scavenging activity of 2, 2- diphenyl-1-picrylhydrazyl (DPPH) assay. Finally, qualitative and quantitative analyses of phytochemical constituents of *Moringa oleifera* leaf extracts were performed by HPLC. Result showed that ethyl acetate extract demonstrated higher antibacterial activity against *Bacillus subtilus* with zone of inhibition 28 ± 8.2 mm, followed by *Streptococcus viridans* (21.67 ± 5.86 mm). These extracts were not active against *E. coli, Klebsiella pneumonia* and *Salmonella* group B. Hexane extract showed antibacterial activity against all tested bacteria. The extracts showed strong antioxidant activity with 50% efficient concentration (EC50) values of 117.94 and 150.96 µg/ml for the methanol and ethyl acetate extracts respectively. The highest phenolic content was observed in methanolic leaf extract with 140.1 9 ± 0. 0.71 (mg GAE/g) while flavonoid was found 98.67±2.10 (mg QE /g) respectively. In addition, different phenolic and flavonoid compounds were also determined individually. This study concludes that *Moringa oleifera* Lam. leaf extracts have significant antimicrobial and antioxidant properties which authenticate its potential as cure against a wide variety of infectious bacterial diseases.

KEY WORDS: MORINGA LEAVES; PATHOGENIC BACTERIA; ANTIOXIDANTS; PHYTOCHEMICALS; ALKALOIDS; FLAVONOIDS.

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INTRODUCTION

Infectious diseases have appeared as one of the major threats to human health around the world and become the major cause of morbidity and mortality. The research of this line has been fruitful and provided medical science with many of the frontline antibiotics in clinical use (Ilanko, et al., 2019). However, antibiotic resistance becomes a problem that continues to challenge the health care sector in a large part of the world. The rise of untreatable bacterial diseases with more resistance to antibiotics and the cause of increasing evolution of multi-drug resistant (MDR) bacteria that remains a wildly unresolved problem and big challenge to health services (Valle Jr, et al., 2015; Maillard, et al., 2020; Tufa, et al., 2020). The discovery of new active compounds against new targets is very important to control the problem that most pathogenic organisms are becoming resistant to antibiotics. Natural antibacterial and antioxidants compounds produced by plants are becoming a big interest in recent research (Dalukdeniya, et al., 2016). They used as safe therapeutics for a wide range of various diseases in medicinal applications (Busani, et al., 2012; Thirumalai, et al., 2018; Adamczak, et al., 2020).

Moringa oleifera leaves are a well-known source of natural antibacterial and antioxidants. For controlling the pathogenic bacteria, *Moringa oleifera* Lam. has become promising natural antimicrobial agent with potential applications in pharmaceutical industry (Reetu, et al., 2020). The extracts of *Moringa oleifera* Lam. can be used to discover antibacterial agent for developing new pharmaceuticals to control various human pathogenic bacteria responsible for the severe illness. *Moringa oleifera* leaves are providing protection against infections and degenerative diseases by inhibiting and scavenging free radicals (Ashour, et al., 2020).

Phytochemical analyses have shown that its leaves are particularly rich in vitamins especially A, D and C. Also, they are containing essential amino acids, antioxidants, flavonoids, and a lot of minerals that are essential for growth and development (Gopalakrishnan, et al., 2016; Su and Chen, 2020). The extracts from *Moringa oleifera* exhibit multiple nutraceutical or pharmacological functions including anti-inflammatory, antioxidant, anti-cancer, hepatoprotective, neuroprotective, hypoglycemic, and blood lipid-reducing functions (Kou, et al., 2018; Shourbela, et al., 2020). Recent trials revealed that *Moringa oleifera* leaves might contribute to prevent obesity as well as obesity-related complications (Mabrouki, et al., 2020). Considering these facts, the present research work was designed to explore the antimicrobial and antioxidant activities of *Moringa oleifera* Lam. leaf extracts. The present study also evaluates the occurrence of natural antimicrobial and bioactive compounds in *Moringa oleifera* Lam. leaf extracts and characterize it to be used as the alternative therapeutic agent. There are only a few elaborative studies on the bioactive constituents of *Moringa oleifera* leaves and their effect on multidrug resistance bacteria. This study aims to bridge the gap. Moreover, much of the evidence remains anecdotal as there has been diminutive concrete scientific studies done to hold authentic claims about *Moringa oleifera* indicating the need of more exploration of this plant (Fahal, et al., 2018; Suresh, et al., 2020).

MATERIAL AND METHODS

Plant Material Collection: Fresh leaves of *Moringa oleifera* Lam. were collected from Saudi Arabia locally and identified in the laboratory by standard flora identification method and confirmed by plant data base https://www.rbg.vic.gov.au/science/herbarium-and-resources/online-databases. The taxonomic identification of this plant also performed by comparison with existing herbarium in Biology department of King Abdulaziz University, Jeddah Saudi Arabia.

Plant Extracts Preparation: The collected *Moringa oleifera* leaves were directly washed to remove debris and allowed to dry under shade. The dried leaves were grounded by blender to fine powder and 790 g was obtained. For the extract preparation, plant material extracted with hexane, ethyl acetate and methanol (72 h each) using a Soxhlet extractor. Extracts were then filtered with Centrifuge at 4000 G for 5 min to remove any debris and concentrated using a rotary evaporator under vacuum at approximately 40°C. The dried extracts lyophilized in lypholyser and stored in airtight tubes at 4°C for further use.

Pathogenic Bacteria Used for Susceptibility Test: A total of 8 bacterial species were tested including four Gram positive bacteria (*Bacillus subtilus, Staphylococcoccus aureus, Streptococcus viridans* and Methicillin resistance *Staphylococcoccus aureus*) and four Gram negative bacteria (*E. coli, Klebsiella pneumoniae, Salmonella* group B, and *Shigella sonnei*) that were obtained from Microbiology Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. These species were originally isolated from the clinical samples and identified based on standard phenotypic tests according to Bergey's manual of systematic bacteriology.

Determination of Antibacterial Activity: Antibacterial activity of the hexane, ethyl acetate and methanol extracts of the plant was studied by standard paper disc diffusion method. Active cultures of eight bacteria were prepared by transferring a sterile loop swap of culture to 5 ml of nutrient broth and incubated at 37 °C for 24 h. The turbidity was adjusted equivalent to 0.5 McFarland units by spectrophotometry at 600 nm. Final cell concentrations were adjusted to 105 $CFUmL^{-1}$ with reference to the McFarland turbidometry (Burt and Reinders, 2003). The positive control was antibiotic kanamycin (25µg/ml) for antibacterial activity. The susceptibilities of the isolated pathogens were determined by the modified Kirby-Bauer disc diffusion method (Bauer, et al., 1966) with Muller Hinton agar plates (MHA, Merck, Germany). Aliquots of inoculums were spread over the surface of agar plates with a sterile cotton swap.

To test the antimicrobial activity, all extracts were dissolved in DMSO to make a final concentration of 400 µl /ml. Each extract (20 ul) was soaked of each extract soaked separately into sterile discs and dried in open air. Solvents were evaporated and then the discs were placed on bacterial cultures. These discs placed on Muller Hinton agar plates, previously swabbed with the bacterial inoculums. The plates were left at room temperature for 1 hour and then the petri dishes were subsequently incubated for 24 h at 37°C. Each experiment was done in triplicate and mean values were taken. Antimicrobial activity was measured in the diameter (mm) of the clear inhibitory zone formed around the disc.

Determination of Minimum Inhibitory Concentrations (MIC): The minimum inhibitory concentration (MIC) of the extracts was determined for most sensitive bacterial species. A 16-hour culture was diluted with a sterile physiologic saline solution (0.9% (w/v) sodium chloride) with reference to the 0.5 McFarland turbidometry to achieve the inoculum approximately equal to 105 $CFUmL^{-1}$ (Burt and Reinders, 2003). In the tube dilution assay, standard bacterial suspension and different concentration of extracts (5, 10, 20, 40, 80 and 160 mg/mL) were added to tubes containing 1 mL Muller Hinton broth. These tubes were incubated at 37°C for 24 hours. The first tube of the series with no sign of visible growth was considered as the MIC. This process has been done three times (Mahboobi, et al., 2006).

Determination of Minimum Bactericidal Concentrations (MBC): To determine the MBC, for each set of test tubes in the MIC assay, a loop full of broth was collected from the tubes without any visible growth and cultured at 37°C for 18 - 24 hours. The highest dilution that yields no colony formation on solid medium was considered as MBC (Motamedi, et al., 2009).

Time-Kill Kinetic Study: The time-kill kinetics was studied by culturing one standard loop of the suspension from the tube possessing MBC on MHA from 0 to 36 hours. This was performed at the first hours of intervals for the first 18-hour study, and then at 2-hour intervals for the later 18 hours (Mahboobi, et al., 2006).

Antioxidant Activity: The antioxidant activity of the hexane, ethyl acetate, and methanol leaf extracts of *Moringa oleifera* was determined based on the free radical scavenging activity of 2, 2- diphenyl-1- picrylhydrazyl (DPPH) according to the method described by Brand-Williams, et al., (1995). One hundred and fifty µl of DPPH solution (4.3mg/3.3ml methanol) was added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. 50µl of various concentrations (25, 60, 120 and 240 µg/ml) of each extract was taken and the volume was made uniformly to150µl using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each, 150µl DPPH was added (to exclude color factor of sample we performed sample blank to each concentration that contains no DPPH, only methanol added, then we subtract the reacted sample with DPPH from blank sample). Absorbance was taken after 15 min at 517nm using methanol as blank on spectrometer. The % scavenging was calculated as: % scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100.

Phytochemical Screening: Preliminary phytochemical screening was performed by using standard tests. Test for alkaloids was done using Dragendroff's test. To 1ml of extract, 1ml of water and 1ml of Aq. NaOH was added. Yellowish brown precipitate was obtained which confirmed the presence of Glycosides. Presence of flavonoids was confirmed by adding 1ml of 10% lead acetate to 1ml of the extract with the appearance of yellowish green precipitate. A froth was obtained by boiling 1ml of extract with 1ml of distilled water which confirmed the presence of saponins. Few drops of 0.1% $Fecl_3$ was added to 1ml extract. A brownish green precipitate was formed confirming the presence of tannins. A brown precipitate was obtained by adding 1ml of $CHCl_3$, 2ml conc. $H2SO_4$ to 1ml of extract indicating the presence of terpenoids (Evans, 2002). To 1ml of extract was added 1ml of 40% NaOH and 2 drops of 1% $CuSO_4$. A pink color was obtained indicating the presence of proteins. Benedict's test was performed with 1ml extract. A slight red precipitate was obtained showing the presence of carbohydrates.

Estimation of Total Phenolic Contents: The total phenolic content of the extracts was determined using the method described by Kim, et al., (2003) with modification. To start the analysis, 1 ml of the extract (0.1 mg/ml) was mixed with 0.2 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 1 ml of 7.6% Na_2CO_3 solution was added to the mixture followed by the addition of 2 ml of deionised distilled water. The mixture was stirred and allowed to stand for 90 minutes. The mixtures (in triplicate) were incubated at 40°C for 30 min and the absorbance was read at 760 nm. The total phenolic content was determined from extrapolation of calibration curve which was made using gallic acid solution and expressed as milligrams of gallic acid equivalents (GAE) per gram of the dry weight.

High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds: High performance liquid chromatography analysis with UV detection was performed for the estimation of the phenolic compounds in the plant extracts. The shaded dried plant material (200 g) was crushed to make it coarse powder. The coarse powder (20 g) was grinded with 25 ml distilled water of 2 N-HCl. The grinded plant extracts were heated in water bath using air condenser at 100°C for 1 h. The plant extracts were filtered using Whatman filter paper No.1. By using a separating funnel, the filtrate was extracted with diethyl ether. The layer of diethyl ether was washed and separated with distilled water and dried over sodium sulphate (anhydrous). The final evaporated extract was obtained using rotary vacuum evaporator at 25°C. The collected extract re-dissolved in HPLC grade ethanol

(5 ml), prior to the injection into HPLC column. The samples were filtered through 0.22 µm organic filter (Millipore) before use (Joshi, 2011).

Table 1. The antimicrobial activity (zone of inhibition) of the tested plant extracts and the tested antibiotic (standard) against pathogenic bacteria by disc diffusion method

Bacteria	Inhibition Zone *mm			
	M	Ea	H	Postive control Kanamycin 25 µg
Bacillus subtilus (c)	9.7±2.52	28±8.2	7±1.73	4.67±0.577
Staphylococcus aureus (C)	0	0	5.67±5.5	6±0
Streptococcus viridans (c)	0	21.67±5.86	8.67±1.53	5.33±0.577
Methicillin-resistant Staphylococcus aureus	4±2	0	8.3±0.58	2.33±0.577
E.coli	0	0	9.3±0.58	5.33±0.577
Klebsiella pneumoniae	0	0	10.67±0.58	5.66±0.577
Salmonella group B	0	0	10.67±0.58	5.66±0.577
Shigella sonnei	0	6±1.73	7±1	5.33±0.577

Values are presented as mean ± SD of triplicate experiments.
Data showed the mean inhibition zone from a triplicate.
c= most sensitive bacteria, C= most resistance bacteria, c= most medium bacteria and 0: No activity.
M: Methanol extract, Ea: Ethyl acetate extract and H: Hexane extract.

Table 1a. Antibacterial activity (MIC and MBC, mg^{-1}) of the plant extracts of *Moringa oleifera* against pathogenic bacteria

Bacteria	Methanol		Hexane		Ethyl acetate	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram Positive						
Bacillus subtilus	10	0	5	40	10	40
Staphylococcus aureus	20	150	0	0	0	0
Streptococcus viridans	15	40	15	40	0	0
MRSA Staphylococcus	30	40	0	0	20	140
Gram Negative						
E. coli	10	20	0	0	0	0
Klebsiella pneumoniae	15	140	0	0	0	0
Salmonella group B	15	20	0	0	0	0
Shigella sonnei	5	150	5	150	0	0

Analysis of Individual Phenolic Acids by HPLC: The reverse phase high performance liquid chromatography (RP-HPLC) analysis was performed for the estimation of phenolic acids compounds like gallic acid, parahydroxy benzoic acid, vanillic acid, syringic acid and ferulic acid. During study, HPLC apparatus was HPLC-Beckman model-322 equipped with 100A model pump, 210 injector, 420 controller, mixer and BD-40 recorder. C18 column (ultrasphere) with specification of 5um (25 cm x 4.6 mm length). Mobile phase was setup strictly with this ratio, methanol: water (1% acetic acid in 20: 80 v/v). Prior to use in HPLC, mobile phase was degased. Flow rate was maintained at 1ml min^{-1} with chart speed 1cm min^{-1}. UV detector was fixed with max 280 nm λ, aufs Attenuation (0.02) and isocratic mode. For individual phenolic compound, the detector response was calibrated and measured with standard phenolic acids strictly as described by Tandon, et al., (2001). All standard phenolic compounds were procured from Sigma-Aldrich chemical company, USA.

Statistical Analysis: The parameters tested in triplicate and the values expressed as the mean ± standard deviation (SD). Statistical analysis was performed with analysis of variance (ANOVA) test and independent sample t-test using the Mega Stat Excel (version 10.3, Butler University) and all columns versus control and the P value < .05 considered as significant.

RESULTS AND DISCUSSION

Moringa oleifera Lam. is reported to have an antimicrobial effect on pathogenic bacteria. The antimicrobial properties of *Moringa oleifera* have been attributed to different parts of the plant, such as the leaves, seeds, pods and stems (Tirado-Torres, et al., 2019) which are known for their antibacterial activity and are counted as rich source of antimicrobial agents (Abadallah and Ali, 2019). The disc diffusion method of antimicrobial susceptibility testing was performed to determine the antibacterial activities of the plant against multidrug resistance pathogenic bacteria through an *in vitro* assessment of finding sensitivity or resistance to an antimicrobial agent (Figure 1). The extracts of *Moringa oleifera* tested showed varying degree of inhibitory activity against the tested pathogens (Table 1).

Comparison of all plant extracts data for their antibacterial potential reveals that ethyl acetate extract showed the highest antibacterial activity with zone size (28 ± 8.2 mm) while the methanol extract exhibited least activity by zone size of (4 ± 2 mm). In the study of Raj, et al., (2011) used different extracts of *Moringa oleifera* Lam. root that tested for antimicrobial activities against some pathogenic bacteria by disc diffusion method and the result showed high antibacterial activity against *Pseudomonas aeruginosa* (18.2 ± 0.2 mm) by ethyl acetate extract.

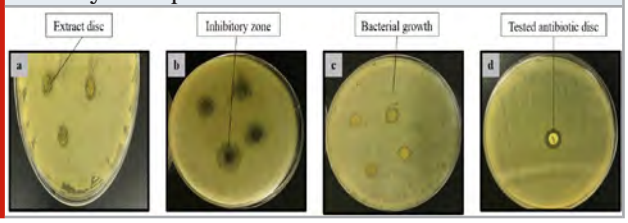
Table 1b. The Time-Kill kinetic of methanolic extract of *Moringa oleifera* at 20 mg/ml concentration against *E. coil*

Hour	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Methanolica extract	+	+	+	+	+	+	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a (+): Growth found
b. (-): Growth inhibition

On the other hand, methanol and ethyl acetate extracts were not active against *E. coli, Klebsiella pneumonia* and *Salmonella* group B. Hexane extract showed antibacterial activity against all tested bacteria. The results were compared with obtained data using standard antibiotics, kanamycin (25 µg/disc that served as reference for inhibition zone diameter. The results of MIC and MBC of hexane, ethyl acetate and methanolic extracts for 8 bacterial species are shown in Table 1a and Time-kill kinetic of methanolic extract of *Moringa oleifera* was 6 hours (Table 1b).

Figure 1: Inhibition zones shown by *Moringa oleifera* extracts (a) Methanol extract against *Bacillus subtilus*, (b) Ethyl acetate extract against *Streptococcus viridans*, (c) Hexane extract against *E. coli*, (d) Tested antibiotic Kanamycin as positive control

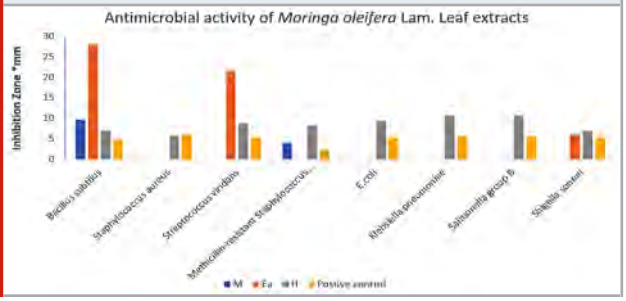


Like this study, Ojiako (2014) showed that ethyl acetate possesses the highest zone of inhibition 10 mm in *Staphylococcus aureus* and *Salmonella typhi* followed by *E. coli* 8 mm, *Candida albican* 4 mm, and *Mucor* 2 mm. Among all tested bacteria *Bacillus subtilus* was more sensitive and showed higher inhibition zone (28 ± 8.2 mm) with ethyl acetate extract, while *Staphylococcoccus aureus* was most resistance bacteria, showed no activity with methanol and ethyl acetate extracts and less zone of inhibition (5.67±5.5 mm) with hexane extract.

Hexane extract showed antibacterial activity against all tested bacteria. The higher zone of inhibition (10.67 ± 0.58) was shown against both *Klebsiella pneumoniae* and *Salmonella* group B and lowest zone of inhibition (5.67 ± 5.5 mm) against *Staphylococcoccus aureus* (Table 1). *Streptococcus viridans*, Methicillin resistance *Staphylococcoccus aureus* and *Shigella sonnei* showed antibacterial activity with two different extracts. Comparing among these bacteria, *Streptococcus viridans* and *Shigella sonnei* showed inhibition zone (21.67±5.86 mm and 6±1 mm) respectively with ethyl acetate extract, while Methicillin resistance *staphylococcoccus aureus* showed less inhibition zone (4±2 mm) with methanol extract. So, *Streptococcus viridans* was the medium sensitive bacteria.

Against *Bacillus subtilus*, ethyl acetate extract showed highest antibacterial activity (28 ± 8.2 mm), while the least activity was (9.7 ± 2.52 mm and 7 ± 1.73 mm) showed by methanol and hexane extracts, respectively. Hexane extract was less sensitive against *Staphylococcoccus aureus* (5.67 ± 5.5 mm) and no activity showed with methanol and ethyl acetate extracts. Also, the ethyl acetate showed antibacterial activity against *Streptococcus viridans* (21.67 ± 5.86 mm) and less activity with hexane extract (8.67 ± 1.53 mm) but no activity with methanol extract. Methanol extract was most resistance against Methicillin resistance *Staphylococcoccus aureus* (4 ± 2 mm) and no activity with ethyl acetate extract, while the hexane extract showed antibacterial activity with zone size 8.3 ± 0.58 mm. Methanol and ethyl extracts showed no activity against *E. coli*, but the hexane extract showed antibacterial potential against *E. coli* (9.3 ± 0.58 mm). *Klebsiella pneumoniae* and *Salmonella* group B showed no activity with methanol and ethyl acetate extracts but high activity in hexane extract with same zone size 10.67 ± 0.58 mm. Ethyl aetate and hexane extract showed activity against *Shigella sonnei* (6 ± 1.73 mm and 7 ± 1 mm) respectively but showed no activity with methanol extract. Similar results regarding antimicrobial activity of Moringa leaves were also reported by (Singh, et al., 2013).

Figure 2: Comparison of antimicrobial potential of the *Moringa* extracts tested by inhibition zone (mm) using Disc Diffusion method. M: Methanol extract, Ea: Ethyl acetate extract, H: Hexane extract and Kanamycin as positive control



The antimicrobial potential of the experimental plant extracts was evaluated by their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with activity of standard, Kanamycin (25 µg). The *Moringa oleifera* leaf ethyl acetate extract was found most effective as compared to the standard Kanamycin 25 µg against *Bacillus subtilus* (28 ± 8.2 mm), *Streptococcus viridans* (21.67±5.86 mm) and *Shigella sonnei* (6±1.73mm). The inhibition zones produced by hexane extracts were more effective than standard against all tested bacteria except for *Staphylococcoccus aureus* (5.67±5.5 mm) which was most resistance bacteria. Methanol extract was most effective as compared to standard against *Bacillus subtilus* and Methicillin resistance *Staphylococcoccus aureus* (28 ± 8.2 mm and 4 ± 2 mm) respectively (Figure 2).

In the study of Yee (2019), different parts of *Moringa oleifera* were tested for the antibacterial activity of five extracts (PE, EtOAc, MeOH, 95% EtOH and H_2O) and were investigated on 5 strains of bacteria which include *Bacillus Subtilis*, *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumalis* and *Escherichia coli* by agar disc diffusion method. Among these five crude extracts, EtOAc (ethyl acetate) extract showed the inhibition zone diameters in the range of 35-45mm has highest antimicrobial activity than the other extracts.

These results support the data of present investigation. Further the resistance and susceptibility testing of our study was quite similar with the results of Abadallah and Ali (2019) showed that ethanol extracts of *Moringa oleifera* leaf demonstrated higher antibacterial activity with average zone of inhibition of 12.49 mm than aqueous extracts (8.00 mm). Based on the susceptibility of the microorganisms to the extracts, *Shigella spp* was found to be the highest susceptible microorganism with average zone of inhibition of 12.46 mm, followed by *Staphylococcus aureus* (11.47 mm), *Salmonella typhi* (10.81 mm), *E. coli* (10.81 mm) while low average zone of inhibition is shown by *Enterococcus faecalis* (9.76 mm) in their study.

Table 2. Absorbance values of *Moringa oleifera* extracts with different conecentration

Methanol zero blank 0.0	DPPH control absorbance at 517nm (0.414)	*DPPH Conc. 1.3mg/ml Methanol*		
Sample concentration	**1µl (25µg/ml)**	**2.5µl (60µg/ml)**	**5µl (120µg/ml)**	**10µl (240µg/ml)**
Mean methanol Abs.	0.377	0.272	0.181	0.038
Mean ethyl acetate Abs.	0.454	0.318	0.271	0.068
Mean hexane Abs.	0.667	0.375	0.345	0.135

Table 3. Antioxidant activity of *Moringa oleifera* extracts

Sample concentration	Methanol fraction	Ethyl acetate fraction	Hexane fraction
1µl (25µg/ml)	8.9%	No inhibition	No inhibition
2.5µl (60µg/ml)	34.3%	23.2%	9.4%
5µl (120µg/ml)	56.3%	34.5%	16.7%
10µl (240µg/ml)	90.8%	83.6%	67.4%

Many other researchers highlighted the positive and effective antibacterial activity of aqueous extracts, chloroform extracts and methanol extracts obtained from leaves, bark and roots of *Moringa oleifera* (Lam.) against four food borne microbial pathogens, *Salmonella enteritica, Vibrio parahaemolyticus, Escherichia coli* and *Listeria monocytogenes*. The main finding of these studies was obtained that all extraction methods showed antimicrobial activity against all tested microorganisms. Lowest and highest antibacterial activity was shown by aqueous extraction and chloroform extraction of residue obtained after aqueous extraction. Highest antibacterial activity was shown by chloroform extraction of residue obtained after aqueous extraction against *Salmonella enteritica*. *Listeria monocytogenes* was found to be the most resistant microorganism to all types of extracts (Dalukdeniya, et al., 2016; Chakraborty, et al., 2019).

The Moringa plant is mainly ascribed to the presence of antioxidant constituents such as phenolic acids and flavonoids. Due to the high concentrations of antioxidants present in *Moringa oleifera* leaves, they can be used in patients with inflammatory conditions, including cancer, hypertension, and cardiovascular diseases. The antioxidants have the maximum effect on the damage caused by free radicals only when they are ingested in combination. A combination of antioxidants found in *Moringa oleifera* leaves was proven to be more effective than a single antioxidant, possibly due to synergistic mechanisms and increased antioxidant cascade mechanisms (Vergara-Jimenez, et al., 2017; Yakoub, et al., 2018). Phytochemical and antimicrobial analysis of *Moringa oleifera* leaf was also screening by Oladeji, et al., (2020).

Figure 3: Antioxidant activity of *Moringa oleifera* extracts M: Methanol extract, Ea: Ethyl acetate extract and H: Hexane extract

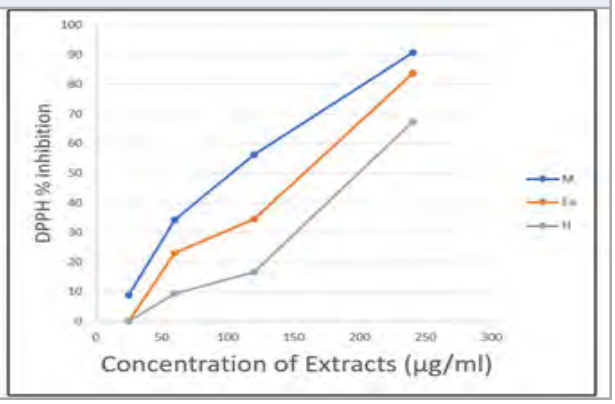


Table 4. Qualitative phytochemical analysis of *Moringa oleifera* leaf extracts

Phytochemical constituents	Extracts Methanol	Ethyl acetate	Hexane
Alkaloids	+	+	+
Glycosides	+	-	+
Flavanoids	+	+	+
Saponins	+	+	-
Tannins	+	+	+
Terpenoids	+	-	+
Proteins	+	+	+
Carbohydrates	+	-	+
Phlobatannins	-	-	+

- indicate absence, + indicates presence

The secondary metabolites in *Moringa oleifera* leaf were extracted by maceration using chloroform, ethyl acetate and ethanol. Some important bioactive metabolites in the leaf extracts, such as steroids, saponins, tannins, flavonoids, terpernoids and phlobatannins were analyzed. The ethanolic leaf extract was observed to show the highest antimicrobial activity when compared to chloroform and ethyl acetate extracts. It also compared favorably to nystatin, streptomycin and gentamicin (standard antibiotics). The study affirmed the therapeutic potency of the plant, indicated by its high antimicrobial effects on some pathogens like *Klebsiella sp, P. aeruginosa, Trichoderma sp, Aspergillus flavus,*

Bacillus cereus, S. pneumoniae, Candida. sp, and *E. coli.*

Quantification of antioxidant activity using DPPH free radical scavenging showed a dose-dependent antioxidant activity for the methanol, ethyl acetate and hexane extracts (Figure 3). From the results obtained, the highest antioxidant activity of 56.3% was exhibited by the methanol extract after 15 min incubation at a concentration of 120 µg/ml. Also, ethyl acetate extract showed good scavenging activity. The extracts showed strong antioxidant activities with EC50 values of 117.94 and 150.96 µg/ml for methanol and ethyl acetate extracts respectively (Table 2). Similar study was reported by Igbo, et al., (2015) using DPPH free radical scavenging for antioxidant activity. From the results obtained, the highest antioxidant activity of 89.1% was exhibited by the methanol extract at a concentration of 125 µg /ml. The extracts showed strong antioxidant activity with 50% efficient concentration (EC50) values of 24 and 44 µg/ml for the ethyl acetate and methanol extracts respectively (Table 3). The study of Fitriana, et al., (2016) provided that *Moringa oleifera* leaves possess antioxidants.

Table 5. Total phenolic (TP) and flavonoids (TF) contents of the *Moringa oleifera* extracts

Extract Samples	Mean Gallic acid Equivalent (mg/g in GAE) Mean	TPC Quercetin Equivalent (mg/g in QE) TFC
Methanol	140.1 9 ± 0. 0.7	98.67±2.10
Ethyl acetate	130.9 ± 0.9	65.77 ± 1.01
n-Hexane	119.4 + 0.5	32.98±2.12

Values are the means ± SD of triplicate

The extracts have been evaluated for its antioxidant activity by 1,1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay and an improved 2,2'-azino-bis- [3-ethylbenzothiazoline sulphonate] (ABTS) radical cation decolorization assay *in vitro*. The methanol extract showed the highest free radical scavenging activity with IC50 value of 49.30 µg/mL in DPPH assay and 11.73 µg/mL in ABTS assay supporting the data of present investigation (Table 2 and 3). Also, the experiments of Vyas, et al., (2020) was clearly indicated that *Moringa oleifera* leaves showed effective free radical scavenging activity which can be attributed to the presence of flavonoids and phenolics along with other compounds.

Preliminary phytochemical investigation showed the presence of diverse phytochemicals which are the bioactive components that can be of use. The result of phytochemicals in the present investigation showed that the plant contains components such as alkaloids, flavonoids, glycosides, tannins, saponins, terpenoids, proteins, carbohydrates (Rodríguez-Pérez, et al., 2015; Udofia, et al., 2020). The phenolic compounds, particularly flavonoids, and terpenoids were abundant in these extracts (Table 4). The total phenolic and flavonoid contents of methanolic extract revealed higher values than ethyl acetate and hexane extracts. The highest phenolic content was observed in methanolic leaf with 140.1 9 ± 0. 0.71 (mg GAE/g) while flavonoid leaf extract was found 98.67±2.10 (mg QE /g) respectively. The result revealed that phenolic content of methanolic extract is higher than that of ethyl acetate and hexane extracts (Table 5). This may be due to different polarity of the solvents used, and phenolics are mainly extracted in higher quantity especially in more polar solvents.

Ethyl acetate extract also showed the good content of phenolic compounds with total phenolic content of 130.9 ± 0.9 mg GAE/g compared to the extract n-hexane that had 119.4 + 0.5 mg GAE/g, respectively (Table 5). The results are in agreement with the findings of Vongsak ,et al., (2013) determined the quantitative analysis of active compounds was accomplished through high-performance liquid chromatography (HPLC). The extract promoted with maximum amounts of total phenolics (13.23 g chlorogenic acid equivalents/100 g extract) and total flavonoids (6.20 g isoquercetin equivalents/100 g extract) also, exhibited high DPPH-scavenging activity (EC50 62.94 µg/ml).Present result indicates satisfactory phenolic contents. Hence they correspond to the results obtained by (Shih, et al., 2011) and (Srivastava, et al., 2020) where the highest total phenolic content was found in the leaves extract of *Moringa oleifera*.

Table 6. HPLC analyses of bioactive compounds of *Moringa oleifera* plant leaf extract

Plant crude extract sample	Phenolics								Flavonoids				
	Gallic acid	Catechin	Epicatechin	Chlorogenic acid	Ellagic acid	Syringic acid	Gentistic acid	Synapic acid	Quercitrin	Isoquercitrin	Quercetin	Kaempferol	Rutin
	105.67 ± 0.01	20.19 ± 0.03	29.73 ± 0.01	79.31 ± 0.02	52.95 ± 0.02	ND	ND	ND	74.90 ± 0.01	75.65 ± 0.02	137.81 ± 0.01	106.75 ± 0.03	60.38 ± 0.02

ND=not detected; concentration of samples is in mg/g
Values represent means ± standard deviation of triplicate readings.

Phenolic compounds have been reported to be an important class of secondary metabolites, found in medicinal plants and used tremendously as a source of anti-infection agent. Nevertheless, they help to reduce the risk of many diseases owing to their antioxidant power (Abdulkadir, et al., 2015; Zhu, et al., 2020). Analyses of individual phenolic and flavonoid compounds by HPLC showed the presence of diverse biochemical constitution. These compounds were identified by the comparison of their retention times and UV spectra to those of authentic standards analyzed under identical conditions. Qualitative and quantitative analyses of *Moringa oleifera* leaf extract depicted that gallic acid, catechin, epicatechin, chlorogenic acid, ellagic acid, quercitrin, quercetin, kaempferol and rutin were detected with different concentrations (Table 6) indicating its medicinal prospective.

The medicinal uses of *Moringa oleifera* may be due to the antibacterial and antioxidant activities of its bioactive phytochemicals particularly phenolic compounds that showed the significance relevance of *Moringa oleifera* in prevention of different diseases by reducing and /or preventing free radicals. This potential may translate into prevention of chronic diseases associated with antibacterial and oxidative stress for humans who consume various parts of *Moringa oleifera* plant (Chhikara, et al., 2020), Also, the work of Rocchetti, et al., (2020) has revealed great abundance of flavonoids and phenolics acids by using different *Moringa oleifera* leaf extracts.

The global emergence of multidrug resistant bacterial strains is increasing, limiting the effectiveness of current drugs and treatment failure of infections. A novel approach to the prevention of antibiotic resistance of pathogenic species is the use of new compounds that are not based on existing synthetic antimicrobial agents. Based on the findings of this study it could be recommended that the extracts of this plant should be further analyzed to isolate the specific antibacterial compounds and defense mechanisms working in it. Speedy clinical trials should be carried out to explore the pharmaceutical potential of the medicinal plants in the treatment of bacterial and fungal infectious diseases.

CONCLUSION

This study concluded that *Moringa oleifera* leaf extracts have antibacterial activity against both Gram positive and negative bacteria, also revealed high potential free radical scavenging activity. The antibacterial activity showed that *Staphylococcus aureus* was more resistant bacteria while, *Bacillus subtilus* and *Streptococcus viridans* were found among more sensitive against all extracts. The highest antioxidant activity of 56.3% was exhibited by Moringa leaves in the methanol extract. This revealed that the leaves contain considerable concentration of antioxidants with good free radical scavenging activity. This result also indicates satisfactory phenolic and flavonoid contents in leaf extracts. It is interesting to conduct more research in depth on Moringa leaves in order that consumers benefit from them as food additive or nutraceutical and biopharmaceutical industries. This study also provides useful information about possibility of discovering new compounds with more effectiveness against multidrug resistance pathogenic bacteria.

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Conflict of Interest: Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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Efficacy and Safety of Umbilical Cord Milking Compared to Deferred Cord Clamping in Term Infants: A Randomized Clinical Trial

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ABSTRACT

Deferred cord clamping (DCC) or umbilical cord milking (UCM) are two proposed methods of placental transfusion for term infants; however, it is still controversial as to which method is the best. We aimed to compare the efficacy and safety of DCC and UCM for term infants and their mothers. We conducted a single center, randomized clinical trial (RCT) of mother-infant pairs delivered at King Abdulaziz University Hospital. Infants were randomly allocated to deferring clamping of the cord for 60 seconds or manual stripping of approximately 20cm of the cord over 2-3 seconds that is repeated three times. Seventy-six infants were allocated to UCM group and 73 to DCC group. There were no significant differences between groups with regards to maternal outcomes. There were no differences in the need for resuscitation, apgar scores at one and five minutes and admission to intensive care unit between groups. Of the enrolled infants, 57% (43/76) and 53% (39/73) of the UCM and DCC groups had completed the study respectively. Apart from a significantly higher Hct in infants allocated to DCC both at 24 hours and at 8-12 weeks, there were no significant differences between the two groups in hematological outcomes. Our results showed the efficacy and safety of UCM compared to DCC in term infants. The adoption of UCM may be considered as an alternative care treatment, especially in cases that are not candidates for DCC.

KEY WORDS: CORD CLAMPING, UMBILICAL CORD MILKING, PLACENTAL TRANSFUSION, TERM, INFANT, NEWBORN.

ARTICLE INFORMATION

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INTRODUCTION

Active management of the third stage of labor has been shown to reduce the risk of postpartum hemorrhage (PPH),(Prendiville et al., 2000), a leading cause of maternal morbidity and mortality.(Bonnar, 2000, Kumar et al.) Clamping the umbilical cord is part of the management procedures of the third stage of labor. (McCann and Ames, 2007) The optimal timing of cord clamping and its effect on neonatal outcomes is an active research area in the past decade. (Mercer et al., 2018) Placental transfusion passively through deferred cord clamping (DCC) or actively through umbilical cord milking (UCM) allows excess residual cord blood to be transferred to neonate rather than going as waste. Both techniques of handling the umbilical cord at birth have been shown to be feasible, effective and safe.(Katheria et al., 2017) In term infants, both UCM and DCC are associated with increased iron storage and reduced risk for anemia compared to immediate cord clamping (ICC).(Alzaree et al., 2018, Das et al., 2018) There was no reported increased risk of hyperbilirubinemia requiring phototherapy or polycythemia from trials comparing UCM or DCC to ICC.(Chiruvolu et al., 2020) Similarly, there was no reported increased risk of maternal complications including PPH.(Panburana et al., 2020).

Maternal and infant conditions for which active management of placental delivery or resuscitation of the neonate are needed preclude DCC. Umbilical cord milking, which is achieved in seconds rather than minutes, allows active transfer of additional blood to the infant at a rapid rate and within a short time without delaying neonatal resuscitation or the active management of placental delivery. In fact, in suspected or pre-empted neonatal asphyxia it can lead to transfer of important stem cells that may help in regeneration of neurons which is an actively researched area.(Katheria et al., 2019) However, there are concerns of acute non-physiological volume overload with UCM. Despite the available evidence that supports the implementation of UCM, it is not part of the international guidelines of handling the UC at birth. The aim of this study was to compare the efficacy and safety of UCM to DCM in term neonates and their mothers.

MATERIAL AND METHODS

We conducted a single center RCT comparing DCC to UCM in term infants. The study was conducted between March 2015 and December 2016 at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The study is registered at the International Standard Randomized Controlled Trial Number (ISRCTN) (Trial ID (ISRCTN15174417)). Moreover, the Biomedical Ethics Unit at King Abdulaziz University Hospital approved this research (HA-02-J-008).

Study population: We included pregnant mothers with anticipated full-term birth at gestational age (GA) > 37 weeks. Gestational age was confirmed by first trimester ultrasonography or was based on solid last menstrual period. We excluded monozygotic twin pregnancy, suspected placenta previa, placental abruption, major congenital abnormality, vasa previa, mothers with Rh sensitization, fetal hydrops, women who had unexplained significant vaginal bleeding as assessed by obstetrician 24 hours prior to delivery, ultrasound diagnosis of umbilical cord knot, and women with known human immunodeficiency virus, or hepatitis virus infections.

Research setting and Study design: We conducted an open parallel-arm RCT. The trial was conducted between March 2015 and December 2016 at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. We identified women who fulfilled the inclusion criteria at the time of admission to the labor and delivery ward, along with their partners, and obtained written informed consent from both parents. All participants were free to withdraw from the research at any given point. he mother-infant pair was randomly allocated to either DCC or UCM group. The allocation was performed using a computer-generated list prepared by an independent biostatistician. Random numbers were generated in 1:1 allocation, without stratification, and the record was kept confidential to primary investigators. The allocation codes were placed in sequentially numbered opaque and sealed envelopes. The envelopes were kept in the delivery room in a lock and key cabinet and were the responsibility of the in-charge nurse at delivery room. The head nurse opened the envelope once she verified that the mother was eligible and had consented to participate in the trial. The obstetrician, pediatrician, and involved nurses were knowledgeable of the intervention because they were required to be present at the time of delivery. Staff members who collected blood samples from infants or subsequent data from medical charts and those who performed the analysis were blinded to which group each infant was allocated.

Intervention: Once the laboring woman consented to the study and delivery was impending, the head nurse opened the envelop with the intervention allocation. Before starting the trial, all medical personnel in the labor and delivery unit received training in the study techniques. Neither DCC nor UCM were practiced in the hospital before the study. In the DCC group, the cord was clamped after 60 seconds. The attending nurse measured the time from full expulsion of the fetus from mother to clamping the umbilical cord using a stopwatch that was available in every room. The delivering staff performed UCM by manually stripping an approximately 20 cm of the umbilical cord over a period of 2-3 seconds and repeated the procedure three times before clamping the cord. For both intervention groups, the delivering staffs were instructed to hold the infant at the level of the introitus in vaginal delivery and on the mother's lap when they were born by cesarean section (CS). The obstetric care was otherwise according to the standard practice at the hospital. The following data were collected from maternal charts (age, parity, diabetes, hypertension, and hemoglobin (Hgb) before delivery). The length of third stage, amount of blood loss and whether there was PPH (blood loss more than 500 cc after vaginal delivery and more than 1000 cc after CS delivery in the first 24

hours after delivery) or need for manual removal of the placenta were recorded after delivery.

Infants were cared for according to the hospital routine. Resuscitation of neonates, when needed, was according to Neonatal Resuscitation Protocol (NRP) and was similar in both groups. The neonatal nurse assessed the infant initially and recorded temperature, heart rate, respiratory rate and blood pressure at one hour of age. Infants were examined daily by the physician on service who was not aware of the allocation intervention as per the clinical routine in the hospital. Nurses collected blood for Hgb, hematocrit (Hct) and bilirubin at 24 hours with the metabolic screening. Clotted blood samples were not repeated. Further blood tests needed were done according to clinical evaluation. Infants were then given an appointment at 8-12 weeks for a follow up and to have their blood samples tested for Hgb, Hct and ferritin. The follow-up appointment was given at the time of the scheduled two months vaccination to make it easy for parents to bring their infants. The parents of the enrolled infants were divided into groups according to the date of birth and the scheduled time for follow up to make it easy for the researcher to communicate with a group of parents using a phone assistive communication application. Parents were approached a month, two weeks and three days before the appointment to remind them with the appointment.

Outcomes: The primary outcome of our study was serum ferritin (ng/mL) at 8-12 weeks of age to evaluate infant's iron status. The secondary outcomes comprised of maternal and neonatal outcomes. Maternal outcomes included mortality, estimated blood loss, PPH (blood loss more than 500cc within the first 24 hours following birth), the need for manual removal of retained placenta, and length of third stage of labor. Neonatal outcomes included apgar score at one and five minutes, the need for resuscitation beyond suction and gentle simulation, NICU admission, hematological parameters at 24 hours of life (Hgb [g/dL], Hct [%]), maximum bilirubin level [umol/L], polycythemia (venous Hct > 65%), need for phototherapy and measures of anemia and iron stores at 8-12 weeks of life (Hgb [g/dL] and ferritin [ng/mL]).

Sample size calculation: A total sample size of 120 infants was required to detect a 30% difference in mean ferritin levels between the two enrolled groups, with a sample power of 80% and significance level of 0.05. Assuming that approximately 20% of infants would be lost due to follow-up issues, a total of 150 infants were required

Statistical analysis: SPSS for Windows, version 21.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Continuous variables were compared using the Student's t test when they were normally distributed and Mann-Whitney U test when skewness in distribution was observed. Chi-square test was used to compare categorical variables between groups. A P value <0.05 was considered significant. Analysis was performed on an intention to treat approach.

Table 1. Demographic characteristics of mother-infants pair

Variable	Delayed cord clamping N=73	Umbilical cord milking N=76	P value
Maternal characteristics			
Maternal age, years, mean (SD)	29.6 (5.6)	29 (6.1)	0.60
Parity, median (IQR)	1 (0,3)	2 (1,3)	0.69
Diabetes, n (%)	7 (9.6)	11(14.5)	0.36
Hypertension, n (%)	1 (1.4)	2 (2.6)	0.58
Cesarean section, n (%)	19 (26.0)	20 (26.3)	0.97
Use of additional uterotonic drugs, n (%)	12 (16.4)	16 (21.1)	0.47
Hemoglobin before delivery, g/dL, mean (SD)	11.4 (1.4)	11.0 (1.4)	0.14
Infant characteristics			
Gestational age, weeks, mean (SD)	39 (1.4)	39 (1.5)	0.75
Birth weight, grams, mean (SD)	3171 (393)	3163 (435)	0.90
Male, n (%)	34 (46.6)	41 (53.9)	0.37

Abbreviations: IQR, interquartile range; SD, standard deviation

RESULTS AND DISCUSSION

One hundred and forty-nine mother-infant pairs were recruited for this study, of which seventy-three were allocated to DCC and seventy-six to UCM. (Figure 1) The demographic characteristics of mother-infant pair are depicted in Table 1. There were no significant differences between the DCC and UCM groups with respect to maternal outcomes. (Table 2) There were no reported maternal deaths in both groups. Studied neonatal outcomes before discharge from hospital did not show significant differences between groups, except for significantly higher Hgb (mean difference, 0.9 g/dL [95% CI, 0.2-1.6 g/dL], p = 0.01) and Hct (mean difference, 2.7% [95% CI, 0.7-4.6%], p < 0.01) at twenty-four hours after birth. (Table 3) Despite repeated reminders, 54.8% (40/73) of infants allocated to DCC and 55% (42/76) of those in the UCM group attended follow up at 8-12 weeks. Reasons for loss to follow-up are shown in Figure 1. While Hct level continued to be significantly higher in the DCC group (MD, 1.8% 95% CI [0.3-3.3%], p = 0.02), Hgb (mean difference, 0.5 g/dL 95% CI [-0.05-1.0 g/dL], p = 0.08) and ferritin (mean difference, 48 ng/ml 95% CI [-19, 116 ng/ml], p = 0.16) were not different between groups among those who were followed. There were no significant differences in baseline characteristics and neonatal and maternal outcomes between those who dropped out and those who completed follow-up. (Table 4).

In this pragmatic, single-center, unmasked RCT of full-term neonates, we identified that there was no difference in maternal, neonatal and infantile outcomes between those who were allocated to DCC for 60 seconds and those whose UC was milked for three times over 2-3 seconds

Table 2. Maternal outcomes

Variable	Delayed cord clamping N=73	Umbilical cord milking N=76	P value
Post-partum hemorrhage, n (%)	1 (1.4)	3 (3.9)	0.33
Manual removal of placenta, n (%)	4 (5.5)	5 (6.6)	0.78
Estimated blood loss, mls, mean (SD)	322 (219)	326 (202)	0.91
third stage of labor Minutes, mean (SD)	4.7 (2.7)	4.7 (4.3)	0.91

Abbreviations: SD, standard deviation

Table 3. Neonatal outcomes in infants who were randomized to deferred cord clamping or umbilical cord milking

Variable	Delayed cord clamping N=73	Umbilical cord milking N=76	Mean difference (95% CI), P value
Outcomes before discharge from hospital			
Need for resuscitation, n (%)	2 (2.7)	2 (2.6%)	0.97
Apgar score at 1m, median (IQR)	9 (9, 9)	9 (9, 9)	0.28
Apgar score at 5m, median (IQR)	10 (10, 10)	10(10, 10)	0.84
Admission temperature, °C, mean (SD)	36.7 (0.19)	36.6 (0.2)	0.03 (-0.4, 0.1), 0.40
Admission systolic blood pressure, mm Hg, mean (SD)	66 (9)	65 (8)	1.4 (-1.5, 4.2), 0.35
Admission diastolic blood pressure, mm Hg, mean (SD)	36 (7.2)	36 (8.5)	-0.2 (-2.8, 2.4), 0.91
Hemoglobin (24h), g/dL, mean (SD)	19.1 (2.2)	18.2 (2.1)	0.9 (0.2, 1.6), 0.01
Hct (24h), %, mean (SD)	53.3(5.9)	50.6 (6.0)	2.7 (0.7, 4.6), 0.01
Bilirubin (24h), umol/L, mean (SD)	95 (33)	91 (34)	4.5 (-6.3, 15.4), 0.41
Maximum bilirubin, umol/L, mean (SD)	115 (55)	110 (58)	5.2 (-13, 23.6), 0.57
Need for phototherapy, n (%)	19 (26.0)	12 (15.8)	0.12
Polycythemia, n (%)	2 (2.7)	0 (0)	0.15
Admission to NICU, n (%)	1 (1.4)	2 (2.6)	0.58
Outcomes at 8-12 weeks			
Hemoglobin, g/dL, mean (SD)	11.5 (1.1) n=37	10.9 (1.2) n=41	0.5 (-0.05, 1.0), 0.08
Hematocrit, %, mean (SD)	33.0 (3.4) n=37	31.3 (3.2) n=41	1.8 (0.3, 3.3), 0.02
Ferritin, ng/mL, mean (SD)	233 (190) n=39	185 (109) n=43	48 (-19, 116), 0.16

Abbreviations: IQR, interquartile range; NICU, neonatal intensive care unit; SD, standard deviation

except for the higher Hct values in the DCC group. In our study, one mother in DCC arm and three mothers in the UCM arm had PPH and the estimated mean blood loss was 322 (±219) and 326 (±202) in DCC and UCM groups, espectively. The mean length of the third stage was similar in both groups and within the normal range. (Magann et al., 2005) Similar finding of no increased risk of maternal complications in the group assigned to UCM was reported in the literature. (Panburana et al., 2020) The incidence of maternal complications is low and thus would require cumulative evidence from several studies to determine safety from maternal aspects. Like other studies, we identified no difference in the need for resuscitation, apgar scores at one and five minutes. admission to NICU and signs of physiologic stability including temperature and systolic and diastolic blood pressure.(Chiruvolu et al., 2020).

Concerns regarding UCM included the fear of active and rapid infusion of large volume of blood to neonates. Though we did not identify any difference in baseline physiological measures between groups, our data should be interpreted with caution as we only included a relatively and potentially healthy group of neonates in our study. This group of neonates as compared to the sicker or more premature group may relatively easily handle an excess of 20-25 ml/kg of volume. Unlike the findings from our study, placental transfusion is reported to increase iron stores at birth and subsequently in early infancy, (Yadav et al., 2015 Das et al 2018). Two key differences may explain these findings. The timing of clamping the UC was deferred for 90 seconds in the DCC group, while milking of the cord was done after clamping the cord at the placental end leaving 25cm of the cord to be milked. (Yadav et al., 2015).

Surprisingly, the Hct level in our study was significantly higher in the DCC group in the first 24 hours of life as well as at 8-12 weeks of age compared to UCM group. The difference in the method used for both interventions could be responsible for the difference in results between studies. The rate and volume of blood transfer in these two methods of placental transfusion have not been studied adequately in the literature. Similar to published trials comparing UCM to DCC in term infants, there was no significant difference between groups in bilirubin level taken in the first 24 hours of life, maximum bilirubin level during stay in hospital, need for phototherapy and incidence of polycythemia. (Chiruvolu et al., 2020).

Table 4. Baseline characteristics and outcomes of participants who completed follow-up and those who were lost to follow-up

Variable	Completed follow-up N=82	Lost to follow-up N=67	P value
Maternal age, years, mean (SD)	30(5.7)	29 (6.0)	0.19
Parity, median (IQR)	1.5 (0,3)	1 (1,3)	0.67
Diabetes, n (%)	9 (11.0)	9 (13.4)	0.65
Hypertension, n (%)	1 (1.2)	2 (3.0)	0.45
Cesarean section, n (%)	18 (22.0)	21 (31.3)	0.19
Gestational age, weeks, mean (SD)	39.2 (1.5)	39 (1.5)	0.40
Birth weight, grams, mean (SD)	3167(399)	3167 (432)	0.99
Post-partum hemorrhage, n (%)	3 (3.7)	1 (1.5)	0.42
Estimated blood loss, mls, mean (SD)	312 (201)	339 (221)	0.44
Need for resuscitation, n (%)	2 (2.4)	2 (3.0)	0.84
Hemoglobin (24h), g/dL, mean (SD)	18.6 (2.3)	18.7 (2.2)	0.72
Hct (24h), %, mean (SD)	51.7 (6.2)	52.2(5.9)	0.60
Bilirubin (24h), umol/L, mean (SD)	91.7 (30.0)	95.5 (37.1)	0.61
Maximum bilirubin, umol/L, mean (SD)	108 (50.4)	118 (63)	0.28
Need for phototherapy, n (%)	15 (18.3)	16 (23.9)	0.40
Polycythemia, n (%)	1 (1.2)	1 (1.5)	0.89
Admission to NICU, n (%)	2 (2.4)	1 (1.5)	0.68

Abbreviations: IQR, interquartile range; SD, standard deviation

We would like to acknowledge the strengths and limitations of this study. The main strength of this study includes masking of outcome ascertainment and clinical care post-placental transfusion. Other crucial aspect of our study was compliance from our obstetric colleagues in attaining a remarkably high success of implementation of allocated intervention. However, loss of follow up was one of the major challenges we faced in this study. Although we had scheduled regular communication with the participants, we had a high rate of attrition. Unfortunately, it is the state of affairs at our institute, which reside in a large metropolitan city encompassing population of differing socioeconomic status.

CONCLUSION

In conclusion, we identified that UCM is effective and safe method of placental transfusion in term infants. However, firm conclusions regarding efficacy could not be made due to significant lost to follow up data for our primary outcome at 8-12 weeks. Umbilical cord milking has the advantage of expeditious handling of the umbilical cord and consequently less resistance of adopting it by the maternal-fetal caregivers in anticipation that resuscitation of the neonate may be needed. However, further research is needed to address the concern about acute and rapid bolus administration in case of UCM rather than gradual equilibration that takes place in DCC. Further, standardization of UCM by specifying the length of the cord to be milked and the rate and the number of times of milking is another area for potential further research.

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Author contribution: H.A. and F.M designed the study. F.M., E.A. and B.E. performed experiment. F.M., E.A. and B.E collected data. H.A, N.B and P.S analyzed data. H.A and F.M wrote the manuscript. N.B and P.S gave technical support and conceptual advice. H.A. critically reviewed the manuscript and supervised the whole study process. All authors read and approved the final manuscript.

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Molecular Identification and Phylogenetic Analysis of Some Rare Actinomycetes Obtained from Al-Lith Hot Spring Area of Saudi Arabia

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ABSTRACT

Isolating microbes from diverse natural ecological units has led to achieve metabolite structural diversity. Hot springs have been less explored ecological sects for discovery of novel microbial bioactive compounds as compared to other terrestrial samples. The capability of thermophilic microorganisms to flourish at high temperatures makes their enzyme systems ideal for various biotechnological applications. In this study, a total of 11 bacterial strains were isolated from Al-Lith hot spring which named Oyun Al-Haar, located about 17 km northeast of Gomika, at about 250 km south of Jeddah, Saudi Arabia. All these isolates were characterized and screened for some enzymes production. They were able to abundantly grow on starch nitrate agar medium and grew optimally at 45^0 with pH 7.0. According to bioinformatics analysis, the eleven bacterial isolates were encompassing 9 actinomycetes and 2 eubacteria namely, *Streptomyces tendae* (3 isolates) and only one isolate for each of *Streptomyces mutabilis, Streptomyces chitinivorans, Streptomyces barkulensis, Leclercia adecarboxylata, Streptomyces fradiae, Streptomyces azureus, Streptomyces macrosporus* and *Enterobacter cloacae*. Based on the enzymatic activities, all isolates were positive for keratinase, gelatinase, chitinase, and lipase. Nine out of the eleven isolates showed protease production in good levels, and out of these11 isolates, 6 isolates exhibited remarkable amylase activity. Bacteria, especially actinomycetes isolated from hot springs area have gained commercial significance as source of thermostable enzymes. There are few reports of enzymes production from the microflora of hot springs, which opens a window for exploring this resource as potential cache of novel strains with bioactive compounds. .

KEY WORDS: Isolating Microbes, Enzymes, Microflora, Encompassing.

ARTICLE INFORMATION

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INTRODUCTION

Secondary products play important roles in several sectors of our society because they are valuable for industrial, biotechnological and pharmacological uses in addition to treat human diseases, such as cancer and bacterial infections (Harvey, 2008). The natural products are largely produced from primary and secondary metabolism of plants and microorganisms (Jose and Jebakumar, 2013). Microbial natural products have made an incredible contribution to the diverse industrial applications (Salehghamari and Najafi, 2016). There are more than 22,000 known microbial secondary metabolites, 70% of which are produced by actinomycetes, 20% from fungi, 7% from *Bacillus* spp. and 1-2% by other bacteria (Subramani and Aalbersberg, 2013). The industrial enzyme market is one of the fastest growing revenue generating sectors in the world. Biocatalysis offers green and clean solutions to chemical processes and is emerging as a challenging and revered alternative to chemical technology. Only 20 enzymes are currently utilized on the industrial level indicating the need for further research and development of low-cost enzymes and their production (Prakash et al., 2013). Enzymes derived from microbial source are generally regarded as safe and they are functional at wide range of temperature, pH, salinity or other extreme conditions (Mukhtar et al., 2017).

Nowadays, the search for new bioactive secondary metabolites has switched to rare genera of actinomycetes from normal habitats or to discovery of strains/species found in unusual habitats. The logic behind these approaches is that such strains may be producers of novel bioactive compounds (Khanna et al., 2011). The unexplored and underexplored environments are promising sources of rare isolates that are believed to be rich sources of interestingly new compounds (Subramani and Aalbersberg, 2013).The extreme habitats are examples of unexplored environments and characterized by chemical or physical conditions that differ significantly from those found in environments that support more abundant and varied life forms (Zhao et al., 2011). Microorganisms have been detected in a variety of extreme environments (Merino et al., 2019).

In recent years, researchers have shown great interest in thermophilic bacteria because of their economic potential, either in useful biological processes such as biodegradation, or in the production of antibiotics and enzymes (Agarwal and Mathur, 2016). Thermophiles including bacterial and archaeal species are found in various geothermally heated regions of the earth such as hot springs (El-Gayar et al., 2017). Hot springs are defined as springs with water at temperatures substantially higher than the air temperature of the surrounding region (Mahajan and Balachandran, 2017). Consistent exposure to high temperatures, high free water content, high moisture content, and a typical chemical composition facilitates the growth of a typical range of hyperthermophilic microorganisms (Mahajan, 2015).

Many microbial strains of following major groups/genera have been reported from hot springs as sporulating and non-sporulating bacilli, actinomycetes and cyanobacteria (Jiang and Xu, 1993). In Saudi Arabia, there are about ten geothermal springs with varying deep temperatures and different flow rates. They are distributed in Gizan and Al-Lith areas (El-Gayar et al., 2017). The aim of the present investigation is the characterization of some thermophilic bacteria, especially actinomycetes isolated from Al-Lith hot spring area, Saudi Arabia in addition to the determination of their potential production of some important enzymes.

MATERIAL AND METHODS

Chemicals and media: Cultivation media and components were obtained from different companies: BDH Limited Poole (England), Biomatic Corporation (Canada), Difco Laboratories (USA), Fluka Chemie (Switzerland), Himedia (India), Holyland (Saudi Arabia), Merck (Germany), and Techno Pharmchem Haryana (India).

Sample collection: Samples from different six locations were obtained from Al-Lith hot spring, Saudi Arabia. The hot spring of Al-Lith which named Oyun Al-Haar (Figures 1) was located about 17 km northeast of Gomika, at about 250 km south of Jeddah at 20˚27'39.4776''N, 40˚28'14.7252''E. The samples (mainly from the area not tampered by human activities) were obtained from: a) soil sediments from surface and at a depth of 1-2 cm, b) water from hot spring, and c) scraping of the inner wall of small channels. The samples were collected aseptically using sterile bottles along with a sterile spatula, transported at 4ºC, and processed directly.

Figure 1.(A): A mapshowing the sampling site atAl-Lith,and (B):The studied hot spring area, Gomika, Saudi Arabia.

Isolation and purification of bacteria: The soil samples were air dried at room temperature for about 7 days prior to inoculation onto isolation plates. Then, soil suspension of each sample was made separately by mixing 1g with 10 ml distilled water and vortexed for 2-5 min, then the mixtures could settle (Istianto et al., 2012). For each soil dilution, about 0.5 ml was taken (Al-Dhabi et al., 2016) and 0.1 ml was taking from hot spring water (Chaudhary and Prabhu, 2016). All these samples were separately spread evenly with a sterile glass rod onto starch nitrate agar medium (Shirling and Gottileb, 1966). Samples from the inner walls of hot spring were directly inoculated on starch nitrate agar medium by cotton swap. The inoculated plates were then incubated at 45°C for a week with presence of humidity (Chaudhary and Prabhu, 2016). After the incubation period, morphologically

distinct colonies were chosen and sub-cultured to obtain pure isolates. The pure cultures were maintained on starch nitrate agar slants at 4^{0}C; for long-term storage, cultures were maintained in glycerol broth (50%, v/v) at -20^{0}C (Uzel et al., 2011). Taxonomic characterization and producing some important enzymes were studied for all isolates.

Taxonomical studies of the isolates: Cultural characteristics of the isolates: Growth capability (heavy, moderate, scanty, or no growth), color of substrate and aerial mycelia, and presence of soluble pigment were examined using various media. These media were International *Streptomyces* Project (ISP) including yeast extract-malt extract agar (ISP-2) (Pridham et al., 1956, 1957), inorganic salts-starch agar (ISP-4) (Küster, 1959), tyrosine agar (ISP-7) (Shinobu, 1958), and starch nitrate agar (Shirling and Gottileb, 1966). The production of melanin pigment was examined using ISP-7 and dark brown to black color pigment means positive result (Srinivasan et al., 2016). In all previous media, the plates were incubated at 45^0 for a week with presence of humidity. The color of substrate mycelia was described by the colors of RAL code (Vishwanatha et al., 2017).

Micromorphological characteristics of the isolates: Light microscope was used to observe the morphology of the isolates. Chitin agar plate was separately inoculated by the isolates and incubated at 45^0 for 7 days to examine by direct microscopic observation at 400X magnification (Caviedes et al., 2000).

Physiological characteristics of the isolates: To determine the optimal temperature and pH of all isolates, the growth capability was observed using incubated the isolates at different temperatures (45, 50 and 55°C) and at various pH (5, 6, 7, 8 and 9). The pH of the media was adjusted by adding 1N NaOH or 1N HCl solution (Al-Dhabi et al., 2016).

Biochemical characteristics of the isolates: Gram staining was carried out by using standard Gram reaction. Antibiotic susceptibility was examined on Muller-Hinton agar (Himedia, India) plates. The isolates were separately spread over the agar plates with disks containing different antibiotics such as amikacin (AK), ceftazidime (CAZ), aztreonam (ATM), piperacillin (PRL), imipenem (IMI), and ciprofloxacin (CIP). After incubation at 45^0 for 24-72 hr., the plates were examined for the presence of inhibition zones around the disc of antibiotic (Aly et al., 2012; Karp et al., 2017).

Molecular identification of the isolates: DNA extraction and PCR amplification: Genomic DNA extraction was performed at King Fahd Medical Research Centre, KAU according to Kumar et al., (2010) with some modifications. The purified isolates were grown for approximately 5 days on starch nitrate agar plates. In a clean tube, bacterial colonies of each isolate were separately mixed with 500 µl of TE buffer. This was followed by 3 cycles of heating in a water bath at 99^{0}C and then treating by liquid nitrogen (3 min in each per cycle). The crushed cells were treated with lysozyme (20 mg/ml) and incubated in a water bath at 37^0 for 30 min. After cell lysis with 10% SDS (w/v) and proteinase K at 55^{0}C for 30 min, the lysate was cooled, extracted for 5 min with a mixture of phenol: chloroform (1:1 v/v) at 10,000 rpm. In the aqueous phase, DNA was precipitated by adding 70-90% ethanol at -20^{0}C for 30 min. After centrifugation, the formed pellet was washed twice with 90% ethanol and dissolved in TE buffer. To obtain RNA free DNA, 20 µl of RNAase solution (20 µg/ml) was added and then incubated at 37^{0}C for 1 hr. The sample was once again extracted with equal volume of phenol: chloroform and precipitated as above. The purity and concentration of DNA was checked using NanoDrop 2000 spectrophotometer (Thermo Scientific).

The 16S rRNA was amplified using a thermal cycler (Applied Biosystems Veriti™ 96-Well Thermal Cycler). Amplification was performed using GoTaq® Green Master Mix, 2X (Promega) and two selected primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'- GWATTACCGCGGCKGCTG -3') (Lane, 1991). PCR conditions consisted of an initial denaturation at 95^{0}C for 5 min; followed by 35 cycles of denaturation (94^{0}C for 40 sec), annealing (58^{0}C for 40 sec), and extension (72^{0}C for 1 min), and a final extension at 72^{0}C for 10 min. The amplification products were examined by 1% agarose gel electrophoresis and the gel was visualized by Ultra-Violet Product (UVP BioSpectrum ® Imaging System).

Bioinformatics analysis: The sequences of 11 isolates were subjected to NCBI BLAST search tool to detect non-chance sequence similarity. BLAST search was restricted to 16S ribosomal RNA sequences, where models (XM/XP) as well as uncultured/environmental samples were also filtered out, such that more reliable results would be attained. Each individual sequence was solely blasted, where the BLAST hit with the lowest expect-value (which indicates the number of non-chance alignments) was picked. In order to ensure that BLAST outputs were governed by expected-value (aka e-value), BLAST algorithm parameter was decreased such the expected threshold was set to more stringent value of 1e–6. Alignment of the 11 sequences was carried out using version 2 of Clustalx (Larkin et al., 2007).

Exploratory data and phylogenetic analyses were carried out under R Project for Statistical Computing (R Core Team, 2019) where exploratory data analysis was done using SeqinR package (Charif and Lobry, 2007). Phylogenetic analysis was carried out by ape package (Paradis et al., 2004). Reconstruction of the phylogenetic tree was done using neighbor-joining method (Nei, 1987). DNA Sequence Polymorphism (DnaSP) (Librado and Rozas, 2009) software was used to analyze the haplotype diversity (Hd), average number of nucleotide differences (Tajima, 1983), the nucleotide diversity (π), polymorphic sites (S), singleton variable sites (SP), parsimony-informative sites (PIP) for each gene, and

the average number of nucleotide substitutions per site between species (Dxy) (Lynch and Crease, 1990).

Screening of the isolates for their extracellular enzymes production: Production of extracellular enzymes by potential isolates was studied by inoculated individually on different agar media at 45^0 for 7-14 days. Bacterial isolates were subjected to screen for amylase, protease, keratinase, gelatinase, chitinase, and lipase activity. For screening amylolytic activity, all isolates were individually grown on starch agar medium (Mohseni et al., 2013). After growth, the plates were flooded with iodine solution. Amylase production was detected as a zone of clearance around the colony on bluish black background due to starch digestion. Iodine forms a bluish black complex with starch but not with hydrolyzed starch (Singh et al., 2019). Indicator of hydrolysis of casein was assessed by growing the isolates individually on skim milk agar medium (Mohseni et al., 2013). Protease production was observed by appearing a clear zone around the colony in the medium (Das et al., 2010).

Regarding the screening for keratinase, feather meal agar medium (Agrahari and Wadhwa, 2010) was individually inoculated with the bacterial isolates. A clearing zone around the growth indicates that this isolate has keratinase. Gelatinase production by the isolates was tested using nutrient gelatin medium in test tubes (Ekpenyong et al., 2016). Partial or total gelatin liquefaction of the inoculated tubes even after exposure to cold temperature of refrigerator (4°C) was considered that these isolates produced gelatinase (Kole et al., 1998).

The bacterial cultures were further examined for their chitinase activity on plates containing chitin agar medium (Kim et al., 2003). The isolates that produced chitinase can grow on the medium and their growth depends, at least partially, on their ability to solubilize chitin. Also, appearance of a clear zone surrounding the colony on a creamish background was regarded that this isolate positive for chitinase activity (Sukalkar et al., 2017; Lacombe-Harvey et al., 2018). To determine lipase activity, all isolated bacteria were individually streaked on solid agar medium with Tween 80 as substrate (Niyonzima and More, 2013). The lipolysis was detected due to occurrence of a zone of clearance around the growth and subsequent formation of white precipitate of calcium monolaurate (Sierra, 1957; Cardenas et al., 2001) because of the combination of released fatty acids and Ca^{+2} ions (Bernal et al., 2015).

Table 1. Cultural characteristics of hot spring isolates grown on starch nitrate agar medium at 45°C for 7 days.

Isolate no.	Growth	*Source of isolation	Color of aerial mycelium	Color of substratemycelium	Soluble pigment
HL1	Heavy	Sediment 1	Pale grey	Terra brown	-
HL2	Heavy	Sediment 1	Grey	Sepia brown	-
HL3	Heavy	Sediment 1	Grey	Terra brown	-
HL4	Moderate	Sediment 2	White	Signal yellow	-
HL5	Heavy	Sediment 2	Pale grey	Golden yellow	-
HL6	Heavy	Sediment 3	Grey	ND	-
HL7	Heavy	Sediment 3	Brown	Brown beige	-
HL8	Heavy	Wall 1	Pale brown	Brown beige	-
HL9	Heavy	Wall 2	Dark green	Olive brown	-
HL10	Heavy	Wall 1	Dark grey	ND	-
HL11	Heavy	Water	Grey	Terra brown	-

*Sediment 1 and sediment 3: at a depth of 1-2 cm; Sediment 2: from surface; -: Soluble pigment absent; ND: Not detected.

Figure 2: Colonies morphology and color of some hot spring isolates grown on starch nitrate agar medium at 45°C for 7 days.

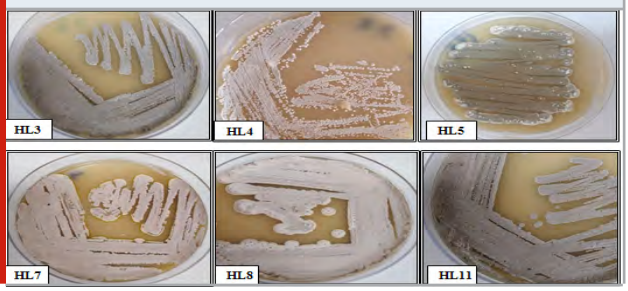
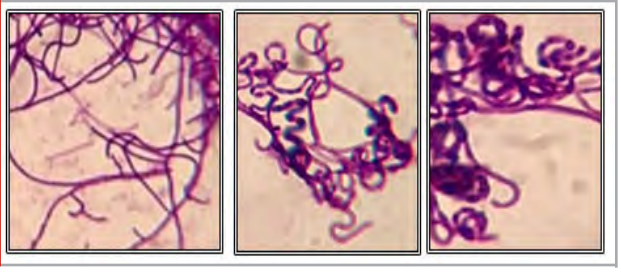


Figure 3: Gram staining of some hot spring actinomycetes under a light microscope at 1000X magnification.



RESULTS

Isolation of actinomycetes: A total of 11 morphologically distinct colonies were obtained from Oyun Al-Haar hot spring using starch nitrate agar medium. The temperature of water in the spring ranged from 46°C to 75°C with pH 5.0-6.0. A total of 7 isolates, named from HL1 to HL7 were obtained from air-dried sediments, while isolates named HL8, HL9, and HL10 were obtained from inner wall of channels, and only one isolate HL11 was obtained from spring water.

Table 2. Some cultural and physiological characters of hot spring bacterial isolates.

Tested character	Isolate no.	Result
Melanin pigment on IPS-7	Isolate HL6	+ ve
	Isolates HL1, HL2, HL3, HL4, HL5, HL7, HL8, HL9, HL10, and HL11	-ve
Growth temperature range	Isolates HL1, HL2, HL3, HL6, and HL11	45-50°C
	Isolates HL4, HL5, HL7, HL8, HL9, and HL10	45-55°C
pH range	Isolate HL5	6.0-9.0
	Isolates HL1, HL2, HL3, HL4, HL6, HL7, HL8, HL9, HL10, and HL11	5.0-9.0

+ve: Positive result; -ve: Negative result.

Table 3. Antibiotic susceptibility of hot spring bacterial isolates to some antibiotics.

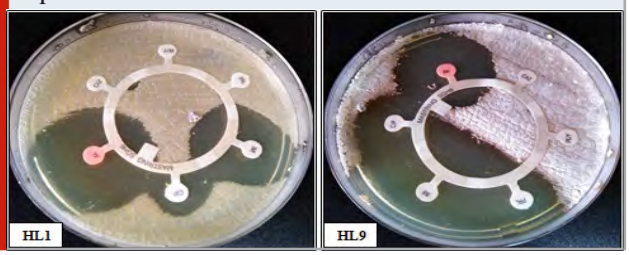
	*Resistance to antibiotics					
Isolate no.	AK	CAZ	ATM	PRL	IMI	CIP
HL1	S	R	R	R	S	S
HL2	S	R	R	R	S	S
HL3	S	R	R	R	S	S
HL4	S	R	R	S	S	S
HL5	S	R	R	R	S	S
HL6	S	R	R	R	S	S
HL7	S	R	R	R	S	S
HL8	S	R	R	R	S	S
HL9	S	R	R	S	S	S
HL10	S	R	R	R	S	S
HL11	S	R	R	R	S	S

*(AK): Amikacin; (CAZ): Ceftazidime; (ATM): Aztreonam; (PRL): Piperacillin; (IMI): Imipenem; (CIP): Ciprofloxacin. R: Resistant (16 mm or less); S: Sensitive (20 mm or more).

Cultural characterization of the isolates: From the result, it was indicated that cultural characteristics of the isolates on starch nitrate agar medium showed various colonies appearance and no soluble pigments were observed in the medium. Also, it was noticed that most isolates (7 isolates) had grey color in different degrees, 2 isolates were brown, one isolate was white and one isolate was green (Table 1 and Figure 2). Colonies characteristics of the isolates on isolation medium can be transformed into different appearance when the organism is sub-cultured on another growth medium. All isolates showed good growth (heavy or moderate) on starch nitrate agar; however, the growth on ISP-2, ISP-4, and ISP-7 plates was varied.

On ISP-2 medium, one isolate HL8 had heavy growth, one isolate HL6 showed moderate growth, 7 isolates HL1, HL2, HL3, HL7, HL9, HL10, and HL11 had poor growth, and isolates HL4 and HL5 showed no growth. On ISP-4 medium, the isolates were divided into two groups; the isolates HL2, HL3, HL6, HL7, HL8, HL9, HL10, and HL11 showed heavy growth and the isolates HL1, HL4, and HL5 showed moderate growth. The growth and melanin pigment production were recorded on IPS-7 (tyrosine agar). On the previous medium, the growth was heavy for isolate HL6, moderate for isolates HL1, HL7, HL8, and HL9, and scanty for these isolates HL2, HL3, HL4, HL5, HL10, and HL11, while melanin pigment was noticed only for isolate HL6.

Figure 4: Antibiotic resistance patterns of the two isolates HL1 and HL9, AK: Amikacin; CAZ: Ceftazidime; ATM: Aztreonam; PRL: Piperacillin; IMI: Imipenem; CIP: Ciprofloxacin.



Micromorphological characterization of the isolates: The morphology of the isolates was examined under a light microscope. All isolates, except isolates HL6 and HL10, were Gram-positive with filamentous hyphae (Figure 3). Isolates HL6 and HL10 were Gram-negative with rod-shaped.

Physiological and biochemical characterization of the isolates: As shown in Table 2, the obtained data from physiological and biochemical examination showed that the optimal growth temperature for all isolates was 45°C. Out of 11 isolates, 6 isolates HL4, HL5, HL7, HL8, HL9, and HL10 grew approximately up to 55°C and the isolates HL1, HL2, HL3, HL6, and HL11 could grow up to 50°C only. It was noticed that all isolates, except isolate HL5, grew well at pH between 5.0 and 9.0 and the pH growth range for isolate HL5 was 6.0-9.0. The optimum pH for all isolates was 7.0. Using antibiotic susceptibility pattern, the growth ability of the isolates was evaluated in the presence of six different antibiotics. All isolates except isolates HL4 and HL9 recorded a clear sensitivity against amikacin, imipenem, and ciprofloxacin with different degrees. Meanwhile, isolates HL4 and HL9 demonstrated resistance against ceftazidime and aztreonam. Table 3 clarified antibiotic susceptibility profiles of all isolates. Figure 4 illustrated antibiotic susceptibility of the isolates HL1 and HL9.

Table 4. Results of NCBI BLAST query for the 11 sequences isolated from hot spring.

Isolate no.	Species	Query coverage (%)	Identity (%)
HL1	*Streptomyces tendae*1	40	99
HL2	*Streptomyces tendae*2	30	99
HL3	*Streptomyces mutabilis*	36	100
HL4	*Streptomyces chitinivorans*	34	97
HL5	*Streptomyces barkulensis*	34	98
HL6	*Leclercia adecarboxylata*	34	97
HL7	*Streptomyces fradiae*	27	100
HL8	*Streptomyces azureus*	90	96
HL9	*Streptomyces macrosporus*	85	100
HL10	*Enterobacter cloacae*	96	95
HL11	*Streptomyces tendae*3	30	99

Bioinformatics analysis of the data: NCBI BLAST query: Table 4 showed results of NCBI BLAST query for the 11 sequenced isolates. The criteria used for query sequence aimed to narrow down the search space (database), such that a smaller the database has more likely to contain the sequence of interest. Therefore, search query was restricted to 16S ribosomal RNA sequences. For all the 11 queries, zero e-values were attained indicated that all alignments were non-chance alignments. The percentages of query coverage ranged from 27 to 96%, where identities % were also high which ranged from 95 to 100%. The results of BLAST query indicated that 11 strains of bacteria encompassing 9 actinomycetes and 2 eubacteria namely, *Streptomyces tendae* (3 isolates) and only one isolate for each of *Streptomyces mutabilis, Streptomyces chitinivorans, Streptomyces barkulensis, Leclercia adecarboxylata, Streptomyces fradiae, Streptomyces azureus, Streptomyces macrosporus* and *Enterobacter cloacae.*

Exploratory data analysis: The base frequencies of the 11 isolates were presented in Figure 5. Base frequencies varied greatly among the 11 isolates, this may be result of great fluctuation in sequence lengths (Figure 6). Sequence length ranged from 499 for *S. azureus* to 1587 base pair (bp) for *S. fradiae* (Table 5). Similarly, GC percentage (%) also varied greatly, where GC% ranged from 49% for *S. fradiae* to 60% for *S. tendae* 2.

Figure 5: Base frequencies of hot spring bacterial isolates.

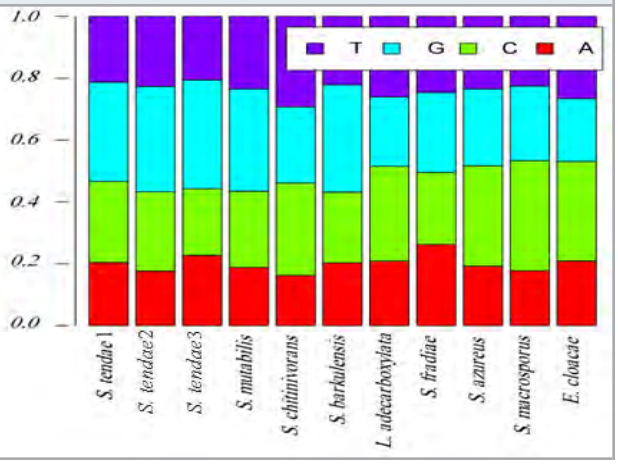
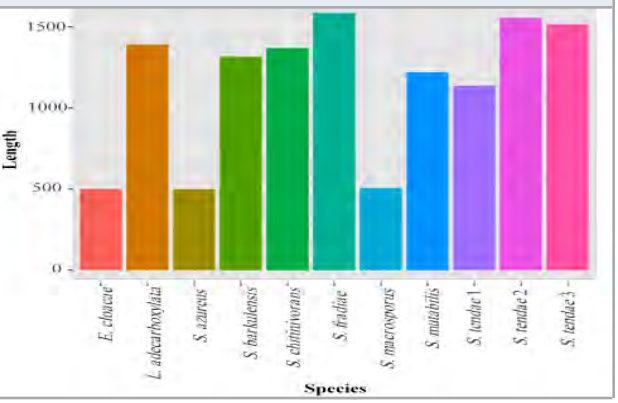


Figure 6: Sequence lengths of hot spring bacterial isolates



Cluster and phylogenetic analysis: Cluster analysis was carried out as pre-processing step to glean an insight into the data distribution. Results of cluster analysis were shown graphically in Figure 7 and Table 6. The resulted dendrogram comprised of 2 large clusters, where *Leclercia* and *Enterobacter* species clustered in one cluster and *Streptomyces* species clustered in the second cluster. The evolutionary history of the 11 isolates was inferred based on neighbor-joining method (Figure 8). The optimal tree with the sum of branch length = 2.5 is

shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were expressed as the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1650 positions in the final dataset.

Table 5. Sequence lengths and GC contents of hot spring bacterial isolates.

Species	Sequence length	*GC content
*S. tendae*1	1138	0.58
*S. tendae*2	1556	0.60
*S. tendae*3	1515	0.57
S. mutabilis	1219	0.58
S. chitinivorans	1370	0.54
S. barkulensis	1315	0.58
L. adecarboxylata	1392	0.53
S. fradiae	1587	0.49
S. azureus	499	0.57
S. macrosporus	506	0.60
E. cloacae	501	0.52

*GC content: Guanine + Cytosine content

Polymorphism and genetic diversity among species: General information about the polymorphisms on the 11 isolates was found in Table 7. The number of sites was 1650, the number of monomorphic informative sites was 296 sites, and the number of polymorphic sites was 175. Of polymorphic sites, 151 sites were parsimony-informative sites (i.e. sites that have a minimum of two nucleotides that are present at least twice) and 24 sites were singletons. The 11 sequences were also analyzed to characterize the sequence diversity. The results of the analysis were presented in Table 8, the haplotype diversity was 1±0.04 and nucleotide diversity was only 0.15, where the average number of nucleotide differences was 68.6. Only one conserved region was found among the 11 isolates in the region between 18 to 552. Measurements of conservation (C)=0.59, homozygosity =0.82 and P-value <0.001. Conservation (C) was calculated as the proportion of conserved site in the alignment region, where homozygosity was measured as 1- heterozygosity.

Screening for enzymes production: From the results (Table 9), it was noticed that all isolates produced keratinase, gelatinase, chitinase, and lipase. Out of 11 isolates, 6 isolates HL1, HL6, HL8, HL9, HL10, and HL11 produced amylase, while isolates HL2, HL3, HL4, HL5, and HL7 showed weak amylase production. On skim milk agar medium, all isolates showed protease production, but the isolates HL1 and HL2 exhibited protease activity in very low levels. Some results for enzymes production were depicted in Figure 9.

Figure 7. Cluster analysis of hot spring bacterial isolates.

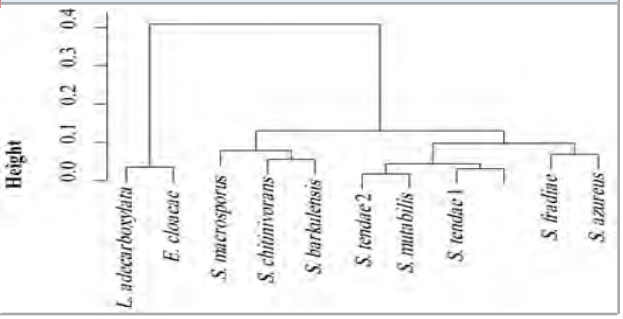


Table 6. Distribution of hot spring bacterial isolates in 3 clusters

Species	Clusters		
	1	2	3
E. cloacae	1	0	0
L. adecarboxylata	1	0	0
S. azureus	0	0	1
S. barkulensis	0	1	0
S. chitinivorans	0	1	0
S. fradiae	0	0	1
S. macrosporus	0	1	0
S. mutabilis	0	0	1
S. tendae	0	0	3

Figure 8: Neighbor-joining phylogenetic tree of hot spring bacterial isolates.

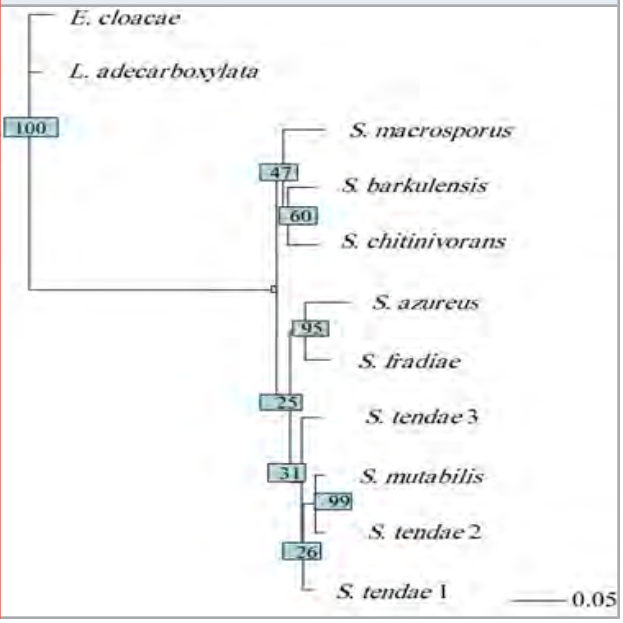


Table 7. Estimated parameters of the polymorphic sites of hot spring bacterial isolates.

No. of sites	No. of monomorphic informative sites	No. of polymorphic sites	Parsimony-informative sites	Singleton variable sites
1650	296	175	151	24

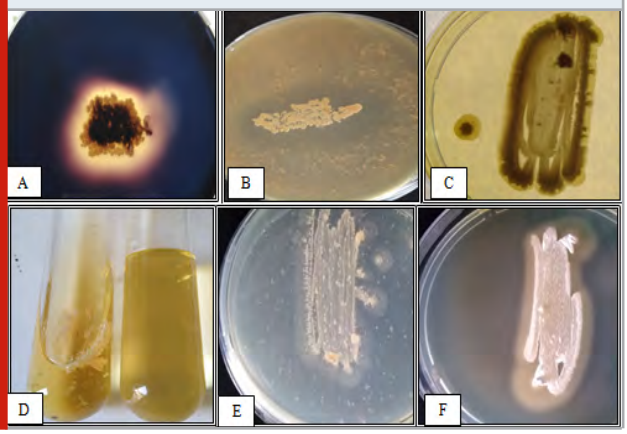
Table 8. Estimated parameters of DNA polymorphism of hot spring bacterial isolates.

No. of Haplotypes	Haplotype diversity±sd	Nucleotide diversityπ	Average number of nucleotide differences
11	1±0.04	0.15	68.6

Table 9. Enzymes production by bacterialisolates.

Enzyme activities	Isolate no.	Enzyme production
Amylase	Isolates HL1, HL6, HL8, HL9, HL10, and HL11	$^{+}$ve
	Isolates HL2, HL3, HL4, HL5, and HL7	-ve
Protease	Isolates HL3, HL4, HL5, HL6, HL7, HL8, HL9, HL10, and HL11	+ve
	Isolates HL1and HL2	-ve
Keratinase Gelatinase Chitinase Lipase	Isolates HL1, HL2, HL3, HL4, HL5, HL6, HL7, HL8, HL9, HL10, and HL11	+ve

Figure 9: Enzymes production by hot spring isolates on different agar media, (A): Starch hydrolysis produced by isolate HL9, (B): A clear zone of skim milk hydrolysis by isolate HL4, (C):Keratinase degradation by isolate HL8, (D):Gelatin liquefaction by isolate HL3, (E): Chitinase production by isolate HL8 and (F):Lipolytic activity of isolate HL2.



DISCUSSION

Factors such as pH, temperature, pressure, salt and nutrient concentrations, water availability, radiation, harmful heavy metals, and toxic compounds are used in defining extreme habitats (Satyanarayana et al., 2005; Procopio et al., 2016). Microorganisms, including actinomycetes, from extreme environments have attracted a great deal of attention, due to their special mechanisms of adapting to the extreme conditions and because they can produce unusual compounds (Meklat et al., 2011). It is postulated that extremotolerants may have larger genetic and metabolic plasticity (Mohammadipanah and Wink, 2016).

Studies on the microbial potential of extreme environments can be utilized to produce novel enzymes that can become future harbingers of green biotechnology (Mukhtar et al., 2017). It is possible to consider thermophiles as sources of industrially relevant thermostable enzymes. Many industrial processes require enzymes such as amylase, cellulase, xylanase, pectinase, protease, and lipase that are operationally stable at high temperatures (Haki and Rakshit, 2003; Bouzaset al., 2006). Thermophilic bacteria and their hydrolytic enzymes have become a chief research subject due to their valuable biotechnological applications (El-Gayaret al., 2017). Thermophilic actinomycetes play an important role in habitats where decomposition of organic matter takes place at elevated temperatures and under aerobic conditions. Using these isolates for direct hydrolysis agro-industrial waste and in biofuel generation also seems a promising strategy (Chaudhary and Prabhu, 2016).

This research aimed to find novel bacteria, especially actinomycetes that produced some useful enzymes from poorly studied extreme habitat, hot spring ecosystem in Saudi Arabia. In this study, a total of 11 bacterial strains were successfully isolated from sediments, walls, and water samples collected from Al-Lith hot spring named Oyun Al-Haar. This spring is located about 17 km northeast of Gomika, at about 250 km south of Jeddah, Saudi Arabia. From the results, it was noticed that numbers of bacteria obtained from hot spring

walls or water were lower than sediment samples. Some previous studies reported similar findings. Khiyami et al. (2012) and El-Gayar et al. (2017) recorded that the total count of bacteria in hot spring water was low. Also, Chaudhary and Prabhu (2016) reported that the sediment sample from hot spring showed a good diversity of actinomycete colonies, while the water sample had only two types. Akmar et al. (2011) found that isolation rate of actinomycetes from hot spring was higher from sediment and biomat than from water sample.

In the current study, taxonomic characterization of the isolates by morphological, physiological, and biochemical characters were studied. Sequencing of the ribosomal RNA gene of bacteria followed by bioinformatics studies were used to identify the bacteria as reported in previous studies (Caverly et al., 2015; Alsanie et al., 2018). In this study, bioinformatics analysis was performed for 11 hot spring isolates to determine the evolutionary and phylogenetic relationships among the isolates. The following strains were identified from the isolates: *Streptomyces tendae* (3 isolates) and only one isolate for each of *Streptomyces mutabilis, Streptomyces chitinivorans, Streptomyces barkulensis, Leclercia adecarboxylata, Streptomyces fradiae, Streptomyces azureus, Streptomyces macrosporus*, and *Enterobacter cloacae*.

The genus *Streptomyces* is classified in the family Streptomycetaceae and has received attention for three main reasons. First, streptomycetes are abundant and important in the soil, where they play major roles in the cycling of carbon trapped in insoluble organic debris, particularly from plants and fungi. This action is enabled by the production of diverse hydrolytic exoenzymes (Barka et al., 2016). Second, the genus exhibits a wide phylogenetic spread (Aderem, 2005). Thirdly, streptomycetes produce a stunning diversity of bioactive secondary metabolites; consequently, they are of great interest in medicine and industry (Hopwood, 2007).

Leclercia adecarboxylata is a Gram-negative bacillus belonging to the family Enterobacteriaceae. It is mainly isolated from environmental or animal specimens but has been recognized as an emerging opportunistic pathogen, with the potential to cause severe infections in immunocompromised patients (Spiegelhauer et al., 2019). *L. adecarboxylata* is a member of the normal gut flora in animals (Hess et al., 2008). *Enterobacter cloacae* is a Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacteria belonging to the family Enterobacteriaceae. Species of the *E. cloacae* complex are widely encountered in nature, but they are also pathogens. *E. cloacae* has taken on clinical significance as opportunistic bacterium and has emerged as nosocomial pathogens from intensive care patients pathogenic, especially to those who are on mechanical ventilation (Mezzatesta et al., 2012).

The bioinformatics analysis results in this study revealed that there was a high variability of sequence lengths between and within species, some species contained long sequences, whereas other species contained short sequences. Furthermore, GC contents were highly variable among the target species. Phylogenetic tree construction of isolated hot spring bacterial strains was done using neighbor-joining method (Nei, 1987). Sequence variations and evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al., 2004). Similarly, same phylogenetic analysis methods were applied by Alsanie et al. (2018) for identification of isolated multidrug-resistant (MDR) bacterial strains.

Molecular identification of the isolates demonstrated low diversity of thermophilic bacteria. Out of 11 isolates, 9 strains were belonging to the genus *Streptomyces*, one isolate was identified as *Leclerciaa decarboxylata*, and another isolate was identified as *Enterobacter cloacae*. Similarly, Aanniz et al. (2015) recorded a very low number of thermophilic bacteria in four hot springs in Morocco. In 2012, Khiyami and coauthors have identified about 15 thermo-aerobic bacteria from Jazan and Al-Lith geothermal springs where *Bacillus cereus, Bacillus licheniformis, Bacillus thermoamylovorans, Pseudomonas aeruginosa* and *Enterobacter* sp. were the dominant strains. The low density of the bacterial populations in hot springs could be attributed to the adverse conditions of these environments or due to the application of culture-dependent identification approaches that identify only a small portion of the total microbial communities (Ranjard et al., 2000). In contrast to the results obtained from this study, 73 strains of bacteria encompassing 8 actinomycetes and 65 eubacteria were isolated and purified from the four sampling points of Vajreshwari-Ganeshpurihot springs, India. The isolates reflected the diversity with respect to macroscopic and microscopic characteristics. Twenty-four were Gram-positive and 49 were of Gram-negative bacteria. About 11% of the isolates were actinomycetes (Pednekar et al., 2011).

Valverde et al. (2012) studied actinobacteria in the hot springs in Zambia, China, New Zealand, and Kenya and observed around 28 major operational taxonomic units (OTUs). They opined that the actinobacterial diversity and endemism were very high in hot spring ecosystems. In addition, several strains of actinomycetes have been previously reported from hot springs (Thawai, 2012; Coman et al., 2015; Kambura et al., 2016). Duan et al. (2014) isolated a novel species, *Streptomyces calidiresistens* from a sediment sample collected from Hehua hot spring in China. Regarding enzymes production in this study, all hot spring bacteria showed positive for keratinase, gelatinase, chitinase, and lipase activity. However, out of the total isolates, only 6 isolates exhibited highly amylase activity and 9 isolates showed good protease production. From hot springs in Gazan, Saudi Arabia, two thermophilic bacteria *Brevibacterium linens* and *Bacillus subtilis* were isolated and showed the capability to produce amylolytic and proteolytic enzymes (El-Gayar et al., 2017).

Another study was conducted with Khalil (2011) who isolated 13 thermophilic bacteria from hot springs in Saudi Arabia. Based on the biochemical characterization, all the isolates were lipase positive, 11 isolates showed

amylase activity, while only 3 of them showed cellulase activity. Bacilli like *Thermus aquaticus* and *Thermus brockianus*, from hot springs have gained commercial significance as source of thermostable enzymes (Brock and Freeze, 1969; Breithaupt, 2001). Thermophilic actinobacteria are biotechnologically important producers of several enzymes such as DNA polymerases, pullulanases, amylases, xylanases, lipases, and proteases (Panda et al., 2017). Chaudhary and Prabhu (2016) isolated species of *Streptomyces* from hot spring water, and these strains produced remarkable amount of thermostable amylase and cellulase with temperature optimum at 55. The thermophilic *Streptomyces* Al-Dhabi-2 strain isolated from hot spring produced various enzymes such as amylase, gelatinase, and deoxyribonuclease (DNase) (Al-Dhabi et al., 2019).

CONCLUSION

In conclusion, hot spring environments are underexplored microbiologically and should not be overlooked for the search and discovery of novel bacteria and their chemical diversity of useful compounds. Studies on unique ecological environments could yield enzymes that could be of great commercial importance in near future. The results of this investigation revealed that Al-Lith hot spring named Oyun Al-Haar was potent source of thermophilic bacteria with hydrolytic enzymes production that can catalyze various reactions at high temperatures. Hence, these isolates can be further evaluated and studied in detail for commercial scale production of enzymes to use in various pharmaceutical and industrial applications. Evidently, more studies on optimal cultivation techniques for thermophiles are required, not only for basic research but also for profiling of their unique microbial products.

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Antimicrobial and Antitubercular Activity of Endophytic Actinobacterium, *Streptomyces sp.* SACC 4 Isolated from the Mangrove Plant *Rhizophora apiculata*

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ABSTRACT

Public health threat due to drug resistant pathogens like *Mycobacterium tuberculosis* and others necessitates the discovery of novel antibiotics. Actinobacteria from understudied sources is emerging as promising source for novel molecules effective against drug resistant pathogens. The present study was undertaken for bioprospecting of endophytic actinobacteria from the mangrove plant *Rhizophora apiculata*. Different actinobacterial cultures isolated from *Rhizophora apiculata* leaves were screened for antimicrobial activity by agar plug method against a wide range of bacterial and fungal pathogens. Antimicrobial metabolites from potential strain SACC4 was produced by agar surface fermentation and extracted using different organic solvents and tested against bacterial pathogens. Antitubercular activity of strain SACC4 was also tested by adopting luciferase reporter phage (LRP) assay. Strain SACC4 was identified based on their phenotypic and molecular characteristics. About 28 distinct actinobacterial strains were isolated from the mangrove leaves in which 50% of the strains showed antimicrobial activity. Notably strain SACC 4 showed broad spectrum activity against bacterial pathogens. Based on the studied phenotypic and molecular characteristics, strain SACC4 was identified as *Streptomyces* species. Among the different solvents tested, methanol extract showed a maximum antimicrobial activity against *S. aureus* in disc diffusion method at 100µg/ml concentration. The methanol extract showed more than 60 percentage inhibition against the standard strain *M. tuberculosis* H37Rv, drug sensitive and MDR *M. tuberculosis* strains at both 100µg/ml and 500µg/ml concentrations. The results of the present investigation revealed that the endophytic *Streptomyces* from mangrove plants are the potent source of novel bioactive metabolites effective against *M. tuberculosis* and other drug resistant pathogens.

KEY WORDS: ACTINOBACTERIA, STREPTOMYCES, ANTIMICROBIAL, ANTI TB, TAXONOMY.

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INTRODUCTION

Antimicrobial resistance (AMR) is a global health crisis. Multi drug resistant pathogens like *Mycobacterium tuberculosis* pose serious threat to public health. Hence, the critical component of AMR solution is the development of truly novel antibiotic. Natural products and their derivatives are mainstays of our antibiotic drugs (Wright, 2017) in which members of the bacterial phylum actinobacteria in particular the genus *Streptomyces* are the known produces of numerous novel secondary metabolites especially antibiotics. However, in recent years, searching of routine sources like terrestrial soil results in the isolation of known actinobacteria which in turn produce known bioactive metabolites (Berdy, 2012). Instead, exploring rare sources like plants may results in the isolation of novel actinobacterial strains with promising bioactive potential. Actinobacteria reside within the healthy plants as endophytes are the promising source for bioactive metabolites to fight against drug resistant pathogens (Golinska et al., 2015, Simpkin et al., 2017). Limited reports on endophytic actinobacetria from plants revealed their enormous bioprospecting potential for clinical, environmental and agricultural applications, (Golinska et al., 2015; Singh et al., 2018).

The present study reports the isolation of endophytic actinobacteria from the mangrove plant *Rhizophora apiculata* and their antimicrobial and antitubercular properties.

MATERIAL AND METHODS

Leaves sample of the mangrove plant *Rhizophora apiculata* was collected from Parangipettai mangrove zone (11.50°N, 79.75°E), Tamil Nadu, India. Endophytic actinobacteria from the leaves were isolated by adopting the method described by Golinska et al., (2015). Morphologically distinct actinobacterial strains were recovered and preserved on ISP2 agar slants at 4°C. All the actinobacterial cultures were screened for antimicrobial activity by agar plug method against clinical strains of *Staphylococcus aureus* MTCC96, clinical strains of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, Candida albicans and *Aspergillus fumigates* (Radhakrishnan et al., 2014). Actinobacterial strain SACC 4 which showed promising activity was selected as potential strain.

Phenotypic and molecular characteristics of strain SACC4 was studied by the methods described by Shirling and Gottileb (1966) and Radhakrishnan et al., (2013). The 16S rRNA gene of SACC4 was amplified by using the primer pairs: 27F 5′AGAGTTTGATCMTGGCTCAG3′ (forward) and 1492R 5′TACGGYTACCTTGTTACGACTT3′ (reverse) and sequencing was carried out at Eurofins Genomics India Pvt. Ltd., Bangalore. The identification of phylogenetic neighbors and calculation of pair wise 16SrRNA gene sequence similarities were achieved using the MEGA version 6 (Tamura et al., 2013) and BLAST analysis (http://blast.ncbi.nlm. nihgov/Blast.cgi). The obtained 16SrRNA sequence was submitted to GenBank to get the accession number.

Effect of solid-state and submerged fermentation on bioactive metabolite production by the strain SACC4 was investigated against *S. aureus* by agar well diffusion method. Effect of solvents on the extraction of bioactive metabolites from the cell free supernatant of strain SACC4 was studied using different solvents such as n-hexane, chloroform and ethyl acetate at 1:2 ratio for 24 hours. The antibacterial activity of different solvent extracts was tested against *S. aureus* MTCC96 by disc diffusion method (Bavya et al., 2011). The antimicrobial activity of methanol extract of strain SACC4 was evaluated against wide range of bacterial pathogens by disc diffusion method at 100 µg/disc concentration. Zone of inhibition was measured after 24 hours of incubation at 37°C and expressed in millimetre in diameter. Effect of different concentration of YEME medium (2X, 1X, 1/2X, 1/4X and 1/10X) on antimicrobial activity of strain SACC 4 was also tested (Yilmaz et al., 2008).

Antitubercular activity of potential strain of SACC4 was studied against standard laboratory strain *Mycobacterium tuberculosis* H37Rv, drug sensitive and multi drug resistant (MDR) clinical isolates of *M. tuberculosis* by adopting LRP assay. The relative light unit (RLU) was measured in a luminometer (Radhakrishnan et al., 2014).

Percentage RLU reduction = Control RLU – Test RLU / Control RLU X 100

Extract showing RLU reduction by 50% or more when compared to control were considered as having anti-tubercular activity.

RESULTS AND DISCUSSION

Totally 28 endophytic actinobacterial cultures were isolated from the *Rhizophora apiculata* leave. Based on their morphology, about 75 % of the cultures were tentatively identified as *Streptomyces* and the remaining 25% are rare actinobacterial strains. Actinobacteria from understudied ecosystems are the promising source for novel bioactive natural products. Limited reports on endophytic actinobacetria from plants revealed their enormous bioprospecting potential for clinical, environmental and agricultural applications (Golinska et al., 2015; Singh et al., 2018). In India, reports on endophytic actinobacteria are very few. In a previous study, Du et al. (2013) analyzed the reported 600 actinobacteria belonging to 34 genera and 7 unknown taxa from 37 medicinal plants in which maximum from root followed by stem and least from leaves. However in the present study 25% of the actinobacterial strains recovered from leaves were found to be rare actinobacterial strains.

In preliminary screening, 18 actinobacterial cultures (64%) showed inhibition against any one of the bacterial pathogens tested. Notably, strain SACC 4

showed promising inhibition against *S. aureus* with 15-18 mm zone of inhibition against *S. aureus, E. coli*, *K. pneumoniae, P. aeruginosa* and the fungi *C. neoformans*.

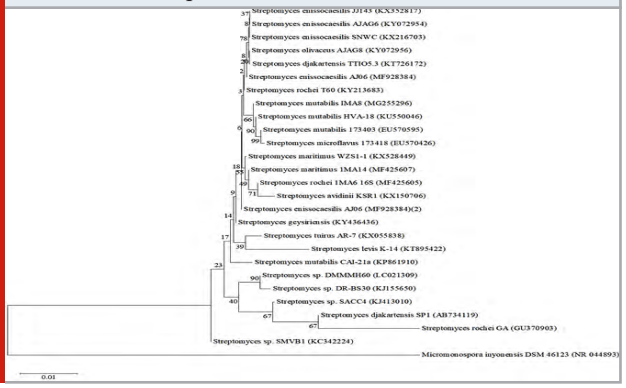
Microscopic, cultural and physiological characteristics of strain SACC4 was given in table 1. PCR amplification and BLAST analysis revealed that the SACC 4 sequence showed 99.9% similarity with the genus *Streptomyces*. Phylogenetic tree constructed based on neighbor-joining method also indicated its closest similarity with *Streptomyces djakartensis* (99%) and other species (Figure 1). The 16s rRNA gene sequence of strain SACC 4 was published in GenBank with the accession number KJ413010. Strain SACC4 was showed 99.8% close similarity with the 16SrRNA sequence of Streptomyces djakartensis. However, certain differences were noted when comparing the physiological characteristics of strain SACC 4 with the characteristics of S. djakartensis (Kounouz et al., 2017). Hence it was assumed that the strain SACC 4 is found to be novel at strain level. In another previous study, it was reported that 85 % of the total 123 endophytic actinobacterial isolates studied were determined to be unique at the strain level (Janso et al., 2010). Further DNA-DNA hybridization and G+C content analysis is needed to confirm its novelty.

Table 1: Characteristics of endophytic actinobacterial strain SACC 4

	Characteristics	SACC4
Micromorphology	Aerial mycelium	+
	Substrate mycelium	+
Cultural characteristics	Fragmentation	-
	Colony consistency	Powdery
	Aerial mass colour	Gray
	Reverse side pigment	-
	Soluble pigment	-
Physiological Characteristics	ISP1 (Tryptone Yeast Extract Agar)	-
	ISP2 (Yeast extract malt extract agar)	+
	ISP3 (Oat meal agar)	-
	ISP4 (Inorganic salt starch agar)	+
	ISP5 (Glycerol asparagine agar)	-
	ISP6 (Peptone yeast Iron agar)	+
	ISP7 (Tyrosine agar)	+
Carbon utilization	Arabinose	+
	Xylose	+
	Inositol	+
	Mannitol	+
	Fructose	+
	Rhamnose	+
Enzyme production	Asparagine	+
	Glutamine	+
PH tolerance	5	-
	6	-
	7	+
	8	+
	9	+
	10	+
	11	+
Temperature tolerance (° C)	20	+
	30	+
	40	+
NaCl tolerance (%)	0	+
	1	+
	2	+
	3	+
	5	-

"+" grown, " – " no growth

Figure 1.The phylogenetic relationship of the potential actinobacterial strain SACC4 based on 16S rRNA gene homology. The tree was constructed using the neighbor-joining method with pairwise-deletion model analyses, which were implemented in the Molecular Evolutionary Genetics Analysis (MEGA), version 6.0 program. The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates. Micromonosporainyonens is was used as out group. Scale bar indicates the number of substitutions per site.



In fermentation study, even until 20 days of fermentation, strain SACC 4 was failed to produce bioactive compounds in submerged fermentation whereas it produced bioactive compounds in agar surface fermentation. The agar plug from ISP2 agar showed activity against *S. aureus* even from 2nd day (11.2±0.72 mm in dm) of fermentation. The zone of inhibition eventually increased from 2nd day to 8th day (17.1±1.09 mm in dm) of fermentation. This is due to the antibiotics production through solid state fermentation are higher quantities and more stable than liquid state fermentation (Subramanian et al., 2012). This finding is in agreement with the antibiotic production by desert soil, Streptomyces strain D25 which showed antibacterial activity and also produced antitubercular pigment only in solid culture (Radhakrishnan et al., 2015).

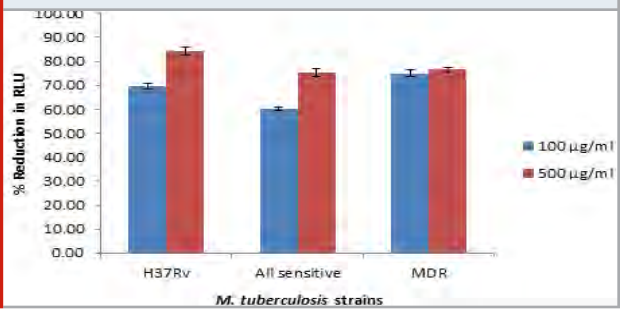
In solid state fermentation, strain SACC4 showed good growth and the methanol extract obtained from the fermented medium showed 18mm inhibition against *S. aureus* but failed to show activity in liquid state fermentation. Isolation of crude extract by solvent extraction is an important phenomenon to find a good solvent that has the potential to extract high yield and most potent bioactive compounds. Our research findings demonstrated that the extract of methanol have significant antimicrobial and antitubercular activity. These results were clearly comparable to that, who evaluated Streptomyces mutabilis which produced an antitubercular activity and antimicrobial activity against gram positive and gram negative pathogens (Mahmoud et al., 2015). Among the different concentrations of YEME agar, 1X concentration of YEME agar was found to support good growth and antimicrobial compound production which was expressed by its activity against *S. aureus* (10.2±0.12), *B. subtilis* (15.0±0.64) and *E. coli* (9.8±0.44). The methanol extract of strain SACC 4 was found to be active against *S. aureus* and wide range of Gram negative bacterial pathogens (Table 2).

Table 2. Antimicrobial activity of methanol extract of endophytic actinobacterial strain SACC 4.

Bacterial pathogens	Zone of inhibition (mm in diameter)*
Staphylococcus aureus	18.2 ±0.15
Klebsiella pneumonia	17.0
Pseudomonas aeruginosa	18.0
Vibrio cholerae 01 ogawa	8.1±0.17
Vibrio cholerae 0139	10.2±0.15
Vibrio parahaemolyticus 03:K6	12.8±0.47
Aeromonassorbia	8.3±0.17
Aeromonashydrophilla	10.2±0.15
Enteropathogenic E.coli 0115	10.2±0.37
Enteroaggregative E.coli 015	10.9±0.35
Enteropathogenic E.coli 0114	10.9±0.21
Salmonella typhimurium	12.0±0.32
S. worthington	12.9±0.38
S. infantis	11.4±0.31
Shigella sonnei	0
S. dysenteriae sero 5	0
S.. boydii sero 1	0
S. flexneri type 2a	0

In LRP assay, the methanol extract of strain SACC 4 was showed more than 60% inhibition against standard laboratory strain *Mycobacterium tuberculosis* H37Rv, drug sensitive and MDR *M. tuberculosis* strains (Figure 2).

Figure 2: Antitubercular activity of potential actinobacterial strainSACC4



Findings of the present study evidenced that mangroves are the promising source for the isolation of bioactive endophytic actinobacteria.

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Effect of *Moringa oleifera* leaf and Flax Seed on Physicochemical and Sensory Characteristics of Chicken Burger

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ABSTRACT

Moringa oleifera is a significant food item which has had huge consideration the 'natural nutrition of the tropics'. *M. oleifera* is very important for its nutritional and medicinal value. Flaxseed are also emerging as a "super food" as more scientific research points to their health benefits. Therefore, in this study, various levels of both M. oleifera and flaxseed were incorporated into chicken burger manufacture to supplement this important food product with their nutritional and therapeutic values. Analyses of the physicochemical and sensory characteristics, phenolic compounds, cooking properties and fatty acids content of burger were conducted. The results indicated that the partial replacement of chicken meat by different levels of FS flour and ML powder gradually increased the total fat and dietary fibers contents from 6% to 9 % and from 1.36 % to 12.47%, respectively. The ash, total phenols and flavonoids content of chicken burger formulations containing FS/ ML markedly increased from 1.8 g/100 g to 3.6 g/100g, 0.0067g/100g to 0.046g/100g and 0.004g/100 g to 0.112 g/100g, respectively. The total unsaturated fatty acid UFA of chicken burger samples increased from 10.018 mg/100g in the control treatment to 20.69 mg/100g in T2. On the other hand, linoleic acid was the most abundant polyunsaturated fatty acids (15.054 mg/100g) found in T1 and T2. The polyunsaturated fatty acid (PUFA)/saturated fatty acid index increased from 0.82 in the control to 1.41 in T1. Various formulations of burger contained appreciable amounts of the macro-minerals as well as the micro-mineral iron. The cooking characteristics of chicken burger were improved while the sensory attributes were slightly decreased. The study concluded that a combination of FS and ML can be utilized as novel fixings to create chicken burger with high nutritive value and organoleptic properties. Future research include investigation of the pharmacological properties of different parts of *M. oleifera* multipurpose tree as well as incorporation in other food items.

KEY WORDS: CHICKEN MEAT, MORINGA OLEIFERA, COOKING CHARACTERISTICS, NUTRITIVE VALUE, CHICKEN BURGER, FLAXSEED.

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INTRODUCTION

Obesity is the excessive or or irregular collection of fat or fat tissue in the body that hinders impairs health through its relationship to the danger of advancement of diabetes mellitus, cardiovascular disease, hypertension, and hyperlipidemia. It is a significant public health epidemic which has progressively worsened over the past 50 years (Kozlov 2019) has experienced stamped dietary changes and quick urbanization in later decades, it was assessed that 26.6% and 10.6% of youths matured 13–18 years are overweight or hefty, respectively (El Mouzan, 2010). A vital sector of the world population has a few confinements to meat and meat products utilization due to saturated fats, cholesterol, salt contents and its relationship with certain sorts of cancer (Cross et al., 2007).

With expanded customer concerns on the utilize of manufactured cancer prevention agents, utilize of common cancer prevention agents in muscle foods is becoming highly relevant in the food industry. Changes in eating propensities emerging from the advancement of society in later decades have led individuals to look for reasonable and more beneficial food with palatable taste and worthy appearance. Hence, the food industry ceaselessly looks for to adjust and crate unused details outlined to progress quality, food safety and shelf life (Selani et al., 2011). A great procedure is to alter the nutritional profile of chicken burger by combining flaxseed and moringa olifera in this way offer choices to those consumers that look for food with a health condition. Numerous herbs and spices are known to contain assortments of phytochemicals, which are potential sources of characteristic cancer prevention agents and antimicrobial compounds that incorporates polyphenols, flavonoids, carotenoids, tannins and phenolic acids (Devatkal et al. 2010).

The *Moringa oleifera* commonly known as drumstick, is local to India, Africa, Arabia, Southeast Asia and South America (Sengupta & Gupta 1970) and customarily being utilized for dietary purposes as vegetable. *M. oleifera* is of extraordinary intrigued in nourishment conservation since in expansion to contributing taste and smell to nourishments, it too contains a assortment of bioactive substances, which are of significant utilize in amplifying rack life.

Lipid oxidation represents one of the most important causes of deterioration in meat and meat products and it affects unsaturated fatty acids particularly polyunsaturated fatty acids (PUFA) in membrane phospholipids as well as cholesterol, mainly low density lipoprotein (LDL) cholesterol. The final end-products of this process can damage the aroma, color, flavor as well as sensorial attributes of meat and allied products; hence reduce the nutritive value [Luna et al., 2010]. Besides nutritional deterioration, lipid oxidation generates cytotoxic and genotoxic compounds which are deleterious for humans health (Botsoglou et al., 2014). The oxidative damage to meat based products results in problems like tissues damaging, purification, loss of nutrients,enhanced free radical generation and malonaldehydes production that reduce the antioxidant capacity of products (Ahn et al., 2009).

The replacement of red meat with chicken in burger production is becoming more popular due to its high fat content and because of no cultural or religious constraints to the consumption of poultry (Mikhail et al., 2014). Among the choices of utilizing nontraditional fixings for the advancement of new poultry products are flaxseed (FS) and Moringa olifera leaves powder (ML). This study amied to ivestigate the effect formulation of combinations of Moringa oleifera and flaxseed on physicochemical and sensory characteristics of chicken burger.

MATERIAL AND METHODS

Chicken meat samples: Chicken meat was prepared as described by Ibrahim et al., 2014. Fresh chicken meat was obtained from a commercial supermarket in Abha, Saudi Arabia , it was washed carefully,deboned , minced using a meat mincer and then chilled at 4±1 for 24 hours before using in processing of chicken burgers. Flaxseed, *Moringa oleifera*, salt, white pepper, black pepper, garlic and onion were obtained from the local market, Abha, Saudi Arabia, and used for preparation of chicken burger.). Treatments were as follows: control = 0%FS + 0% MLP; T1 = 20%FS + 0% MLP; T2 = 5%FS + 15% MLP; T3 = 10%FS + 10% MLP; T4 = 15% FS + 5% MLP; and T5 = 0%FS + 20% MLP.

Preparation of chicken burger: Fresh chicken burger samples were prepared as described by Table (1). All ingredients were minced twice, after mincing, 50 gram portions were shaped into burger using a burger– forming machine to obtain round discs 8.1 cm diameter and 0.5 cm thickness. The burger samples were cooked for 20 min in pre-heated hot air oven at 180± 1^{0}c to an internal temperature of 75 °c. To ensure uniform cooking, the burgers were turned over at 10 min interval.

Table 1.Ingredients (%) of five blends of chicken burgers formulated with flaxseed flour and *Moringa oleifera* leaf

Ingredients (%)	Control	T1	T2	T3	T4	T5
Chicken meat	81	61	61	61	61	61
Cold water	15.40	15.40	15.40	15.40	15.40	15.40
Salt	1.0	1.0	1.0	1.0	1.0	1.0
White pepper	0.2	0.2	0.2	0.2	0.2	0.2
Black pepper	0.2	0.2	0.2	0.2	0.2	0.2
Garlic powder	0.2	0.2	0.2	0.2	0.2	0.2
Onion powder	2.0	2.0	2.0	2.0	2.0	2.0
Flaxseed flour	0	20	15	10	5	0
Moringa oleifera powder	0	0	5	10	15	20

Chemical analysis: Determination of the contents of moisture, protein, fat and ash were determined according to AOAC (2000) methods. Moisture content by using the air oven drying method , the protein content by the Kjeldahl method, while the fat content by the Soxhlet method.

Determination of total phenols content: Total phenolic contents of samples were determined according to the method (Boyer & Hai Liu, 2004). One ml of extract was mixed with 5 ml of 10 % Folin-Ciocalteu reagent in distilled water and 4 ml of 7.5 % sodium carbonate solution. The samples were maintained at room temperature for 30 min, the absorbance at 765 nm (UV-VIS spectrophotometer, Apel, Japan) was measured. The calibration curve was constructed within the concentration range 0.075–0.6 mg/ml of gallic acid. Means were calculated from three parallel analyses as gallic acid equivalents in g/100 g of dry plant material using the following equation: C = a× γ× (V/m) ×100,
C: total phenols g/100g as gallic acid; a: dilution number; γ: concentration obtained from calibration curve (mg/ml); V: volume of extract (ml); m: weight of sample (g).

Estimation of total flavonoids: Flavonoid contents of samples were determined according to the method described by Lamaison and Carnat (1990) using AlCl3.6H2O with slight changes. A stock solution of 1 mg/ml of quercetin (standard) was prepared using 50% methanol solvent, and the previous samples' stock solutions were used. Forty microliters of samples and quercetin (of six different dilutions) were separately mixed with 200 µl 2% (w/v) AlCl3.6H2O. The mixture was incubated for 10 min at ambient temperature, and absorbance values at 440 nm were obtained. Calibration curve for quercetin standard was plotted. Regression equation of the curve, absorbance value = 0.0401 (quercetin concentration)–0.0017 with R2 value, 0.9992 was obtained. The equation was used to calculate the quercetin content in 1 g of sample (mg quercetin equivalence (QE)/g of sample.

Physical chrateristics of burger: Weight losswas calculated by the differences in weight between uncooked and cooked burgers, divided by the weight of uncooked burger. Diameter was calculated by the differences in diameter between uncooked and cooked burgers, divided by the diameter of uncooked burger. pHwas measured by pH meter.

Minerals analysis: For determination of mineral elements contents (Inuwa et al., 2011) was employed. These elements incudedcalcium, magnesium, potassium, sodium, phosphorous and iron. The mineral solution obtained from ashed materials wasfiltrated and used to determine mnerals contents using Varian Spectra AA atomic absorption spectrometer.

Determination of cooking properties: The cooking yield was determined as reported by Murphy et al. (1975) as follows: Yield of cooked burger =

$$\frac{\text{weight of cooked burgers}}{\text{weight of raw burgers}} \text{ X } 100$$

Fat retention was calculated according to Murphy et al. (1975) as follows: Fat retention = cooking yield

$$\text{X} \frac{\text{\% fat in cooked burgers}}{\text{\% fat in raw burgers}}$$

Based on the method of El- Magoly et al., (1996), the moisture retention was determined as follows: Moisture retention = cooking yield

$$\text{X} \frac{\text{\% moisure in cooked burgers}}{\text{\% moisure in raw burgers}}$$

Dimensional shrinkage: The dimensional shrinkage (DS) was calculated according to the formula of Murphy et al., (1975) as follows:

$$DS = \frac{(\text{raw thickness} - \text{cooked thickness}) + (\text{raw dimeter} - \text{cooked dimeter})}{\text{raw thickness} + \text{raw dimeter}} \text{X } 100$$

Where DS: Dimensional Shrinkage

Sensory evaluation: Twenty panelists from the students of Home Economic Faculty, King Khalid University, Saudi Arabia, conducted the sensory tests using a 5-hedonic scale test according to Pimentel et al., 2016). The panelist were given a hedonic questionnaire to test the taste, flavor, juiciness, tenderness, texture, color and overall acceptability of coded burger samples. The samples were scored on a scale of 1 – 5 (1 = poor, 2 = fair, 3 = good, 4 = very good, 5 = excellent).

Determination of fatty acids content: The fatty methyl esters were analyzed in a Shimadzu Model GC-QP-2010, injection volumes were 1µl/sample. Methyl esters of the different samples were identified by their relative retention times compared to those of the reference standards (GLC-68, NuChek Prepn. Inc., Elysian, MN) and quantified by their relative peak areas. Oil-freshly chicken meat was extracted from sample (1g) using petroleum ether as solvent. The solvent mixture was evaporated to dryness under nitrogen and then transesterified with sulfuric acid in the presence of methanol for 3h at100°C. The resulting fatty acid methyl ester was run through a column containing $MgSO_4$ plus silica and evaporated again to dryness by heating the solution to 60°C while flushing with nitrogen.

The fatty methyl esters were re-dissolved in 1-2ml of hexane and analyzed in a Shimadzu Model GC-QP-2010, injection volumes were 1µl/sample. Methyl esters of the different samples were identified by their relative retention times compared to those of the reference standards (GLC-68, NuChek Prepn., Inc., Elysian, MN) and quantified by their relative peak areas.The oils were converted to methyl esters by transesterification

in methanol with hydrogen chloride catalyst. The esters were examined by GLC and the composition was estimated by measuring the peak areas. The GLC analyses were made using a copper column at 185' and a thermal conductivity (thermistor) detector. The liquid phase was a diethylene glycol - succinic acid polyester.

Statistical analysis: Results were analyzed using analysis of variance (ANOVA) using the SPSS statistical package program, and differences among the means were compared using the Duncan's Multiple Range test. At a significance level of 0.05 was chosen to evaluate different chicken burger samples.

RESULTS AND DISCUSSION

Effect of flaxseed flour and *Moringa oleifera* leaf powder on chemical composition of chicken burger.The effect of flaxseed flour (FS) and Moringa oleifera leaf powder (ML) on chemical composition of chicken burger is shown in Table (2). The partial replacement of FS and ML in chicken burger gradually increased their content of total fat and dietary fibers contents from 6% to 9 % and from1.36 % to 12.47%, respectively, as shown in Table(2). Also it could be noticed that protein and moisture contents of chicken burger supplemented with flaxseed flour (FS) and moringa leaf powder (ML) gradually decreased from 85.2 g/100 g to 56.75 g/100 g) and from 80g /100g to70g/100g, respectively with increasing FS/ML levels, as compared with those of the control sample.

Moreover, it could be noticed that ash, total phenols and flavonoids content of chicken burger formulations containing FS/ ML markedly increased from 1.8 g/100 g to 3.6 g/100g, 0.0067g/100g to 0.046g/100g and 0.004g/100 g to 0.112 g/100g, respectively. Mahima, et al., (2014)reported that the leaves of *Moringa olifera* contained moisture 72.39%, ether extract 2.525 %, crude protein 14.125%, crude fiber 23.09%, total ash 9.15%, nitrogen free extract 51.11%, cellulose 11.0% hemicellulose 10.24% and lignin 2.41%. An analysis of brown Canadian flax averaged 41% fat, 20% protein, 28% total dietary fiber, 7.7% moisture and 3.4% ash, which is the mineral-rich residue left after samples are burned (Morris et al., 2011).

Generally these results are in agreement with those obtained by A.ElifBilek & SadettinTurhan (2009) who concluded that fat and ash content of raw patties increased, while moisture and protein content decreased with increased flaxseed flour. The same trend (except fat content) was also observed after cooking. The addition of flaxseed flour did not affect pH values of raw and cooked beef patties. Nahla, (2014) reported that Beef burger is one of the foremost favorable foods in hotels and fast food merchants. Moringa leaves is rich source of antioxidants and biocompounds that have numerous parts in aniscipatig numerous illnesses. Johnsson, et al., (2002) detailed that phenolic compounds in flaxseed may work as blocking or trapping agents for chemically actudated cancers caused by fragrant carcinogens.

Table 2. Effect of flaxseed flour and *Moringa oleifera* leaf powder on chemical composition of chicken burger (mean D.W)

Samples	Fat (g/100g)	Moisture (g/100g)	Ash (g/100g)	Protein (g/100g)	Fiber (g/100g)	Phenols (g/100g)	Flavonoids (g/100g)
Control	6.0+0^{d}	80+1.15^{a}	1.8+0.2^{c}	85.2+ 0.006^{a}	1.36+ 0.006^{f}	0.0067+ 0.0003^{f}	0.004+ 0.001^{e}
T1	6.3+0.3^{c}	78+2.08^{b}	3.0+0.58^{ab}	52.62+ 0.006^{d}	12.12+ 0.006^{b}	0.011+ 0.0003^{e}	0.044+ 0.001^{d}
T2	6.8+0.42^{c}	75+2.89^{b}	3.0+0.0^{b}	57.92+ 0.012^{b}	12.47+ 0.006^{a}	0.023+ 0.00^{d}	0.06+ 0.0003^{b}
T3	7.5+0.5^{b}	73+3.0^{c}	3.27+0.41^{a}	55.78+ 0.006^{c}	10.59+ 0.003^{c}	0.027+ 0.0001^{c}	0.06+ 0.0^{b}
T4	8.0+0.58^{b}	72+1.15^{c}	3.3+0.38^{a}	55.5+ 0.003^{c}	7.72+ 0.006^{d}	0.046+ 0.0^{a}	0.112+ 0.001^{a}
T5	9.0+0.35^{a}	70+1.53^{c}	3.6+0.4^{a}	56.75+ 0.006^{b}	6.01+ 0.0^{e}	0.038+ 0.001^{b}	0.055+ 0.0003^{c}

Effect of flaxseed flour and *Moring oleifera* leaf powder on fatty acid profile (mg/100g) of chicken burger: The effect of partially replacement chicken meat burger with flaxseed flour and M. oleifera leaf powder on saturated fatty acids (SFA) and unsaturated fatty acids (USFA) contents of produced chicken burger was shown in Table (3). From the table, it could be noticed that total saturated fatty acid of chicken burger samples was in the range of 12.193 to 32.325 mg/100g. However, the higest fatty acids found in (T5) burger were Magaric acid (11.801 mg/100g) and tridecylic acid (6.638 mg/100g). Moreover, myristic, pentadecylic acid and arachidic fatty acids were also present in significant amounts (4.74, 1.687 and 1.091 mg/100g, respectively). Mean while, total unsaturated fatty acid UFA reached 10.018 mg/100g and linoleic acid (5.931 mg/100g) was the most abundant polyunsaturated fatty acids. While the contents of oleic and linolenic acids were 3.841 mg/100g and

0.246 mg/100g, respectively, the identified UFA content gradually increased in different chicken burger samples supplemented with SF and MLP.Whereas the increase in monounsaturated fatty acids (MUFA) oleic acid and polyunsaturated fatty acids (PUFA)linoleic acid and linolenic acid contents for those different chicken burger formulations was 25.61 %and 154.88%, respectively when compared with control formula.

Kang et al., (2005) indicated that it is a desirable to maintain to a p/s ratio(polyunsaturated/saturated fatty acids) approximately (1.0-1.5) with an ideal pI value (app. 80-90) in the diet in order to reduce the risk of cardiovascular disease (CDV) and oxidative stress. The ratio of unsaturated and saturated fatty acids content is expressed as U/S index .From Table (3) the U/S ratio of formulated chicken burgers, especially in burger formula (T1 and T2) were the highest U/S index (1.41 and 1.22) when compared with values of other burger formulation. The ratio of unsaturated and saturated fatty acids content is expressed as U/S index. WHO, (2015) reported that it is important the ratio U/S value be higher than 1 due to the essential character of the linoleic fatty acid.

Table 3. Effect of flaxseed flour and *Moringa oleifera* leaf powder on fatty acid profile (mg/100 g) product of chicken burger

Fatty acid	Control	T1	T2	T3	T4	T5
Caprylic acid (C8:0)	N.D	N.D	N.D	N.D	0.108	0.318
Pelargonic acid (C9:0)	0.046	N.D	N.D	N.D	N.D	N.D
Capric(10:0)	N.D	N.D	N.D	N.D	0.163	0.446
Undecylic acid)C11:0)	0.165	N.D	N.D	0.085	0.464	1.369
Tridecylic acid (C13:0)	0.908	N.D	N.D	0.456	2.436	6.638
Myristicacid (C14:0)	0.657	N.D	N.D	0.332	1.738	4.749
Pentadecylic acid (C15:0)	0.253	N.D	N.D	0.114	0.605	1.687
Palmitic acid (C16:0)	0.407	0.787	0.787	0.434	0.284	0.592
Margaric acid (C17:0)	9.757	7.509	7.509	4.815	4.164	11.801
Oleic acid (C18:1)	3.841	4.825	4.825	2.72	2.145	3.901
Linoleic acid (C18:2)	5.931	15.054	15.054	8.006	5.992	9.583
Linolenic acid (C18:3)	0.246	0.69	0.69	0.298	0.234	0.375
Nondecylic acid (C19:0)	N.D	5.565	5.565	2.183	1.433	0.314
Arachidic acid (C20:0)	N.D	0.859	0.859	1.257	N.D	1.091
Heneicosanoic acid (C21:0)	N.D	N.D	N.D	N.D	0.098	N.D
Behenicacid (C22:0)	N.D	2.024	2.024	2.545	3.011	3.321
Saturated fatty acids (SA)	12.193	14.72	16.744	12.221	14.504	32.325
Unsaturated Fatty acids (US)	10.018	20.089	20.569	11.024	8.371	13.859
U/S index	0.82	1.41	1.22	0.95	0.577	0.448

Means with the same letter are not significantly different (P= 0.05)

Table 4. Effect of flaxseed flour and *Moringa oleifera* leaf powder on mineral content of chicken burger

samples	Mg (ppm) Fresh weight	Fe (ppm) Fresh weight	P (ppm) Fresh weight	Na (ppm) Fresh weight	K (ppm) Fresh weight	Ca (ppm) Fresh weight
242.812^{d}	12.385^{c}	2102.850^{c}	7687.39^{b}	3555.54^{d}	291.694^{d}	Control
726.286^{c}	20.185^{c}	3158.040^{a}	7857.70^{b}	4682.16^{c}	589.784^{c}	T1
734.718^{c}	17.557^{c}	2893.145^{a}	7312.56^{c}	4820.92^{c}	1293.243^{b}	T2
1099.084^{b}	94.063^{b}	2592.382^{b}	8490.42^{a}	5628.51^{a}	2711.197^{a}	T3
1307.998^{a}	126.843^{a}	2381.640^{bc}	6982.20^{d}	5013.28^{b}	3654.172^{a}	T4
1442.675^{a}	128.741^{a}	2137.906^{c}	7823.46^{b}	5402.07^{ab}	3654.172^{a}	T5

Means with the same letter are not significantly different (P= 0.05)

Effect of flaxseed flour and *Moringa oleifera* leaf powder on mineral content of chicken burger: Table (4) presents the mineral composition of chicken burger formula: calcium content of T4 and T5 were higher than the control that of the control which was 291.694 and 3654.172 ppm,respectively. In addition, Table (4) reveals that formula (T5) was higher in Fe, Mg compsred with the control formula. Phosphorus of formula (T1) was

3158.040 ppm, which is more than 50 % in the control sample. Potassium and sodium of formula (T3) were 5628.51 ppm and 8490.42 ppm, respectively, which are more than 58% and 10.44% respectively in control sample.

Lakshmipriya & Kumar, (2016) reported that Moringa has lot of minerals that are essential for growth and development among which calcium is considered as one of the important minerals for human growth while 8 ounces of milk can provide 300-400mg, Moringa leaves can provide 1000 mg and moringa powder can provide more than 4000 mg. beef has only 2 mg of iron while Moringa leaves powder contain 28 mg of iron. Singh et al., (2011a) reported that flaxseeds are source of minerals as calcium, mangnesium and phosphorus. It is of great importance, being that a 30 portion of the seed constitutes 7 % to 30 % of the recommend dietary allowances (RDAs) for these minerals. Rockwood et al., (2013) stated that Moringa provides 17 times more calcium than milk, 15 times more poatasium than bananas and 25 times more than spinach.

Effect of flaxseed flour and *Moringa olifera* leaf powder on cooking properties of chicken burger: The cooking characteristics of chicken burgerare depicted in Table (5). The addition of flaxseed flour and Moringa olifera leaf powder to chicken burger significantly ($P \leq 0.05$) affected cooking characteristics of chicken burger. Data represented in Table (5), indicated that the control sample showed a significant decrease in cooking yield when compared with other treatments in all cases. Furthermore, the treatment T5 (at 0% flaxseed flour +20% *Moringa olifera* leaf powder) showed significant increase in cooking yield when compared with other treatments. Moisture retention was significantly ($P \leq 0.05$) high in all treatments compared to control samples. The improvement in moisture retention of the patties may be attributed to increases in the water absorption capacity of heated protein flours, the heat dissociation of proteins, the gelatinisation of starch in the flour and the swelling of the legume fibre (Modi, et al., 2004).

Table 5. Effect of flaxseed flour and *Moringa olifera* leaf powder on cooking properties of chicken burger

Samples	Cooking yield (%)	Fat retention (%)	Moisture Retention (%)	Dimension Shrinkage (%)
Control	60+0^{e}	56+3.06^{c}	54.01+0.82^{c}	22.09+0.003^{a}
T1	72+0^{b}	68.87+7.60^{a}	67.46+1.78^{a}	12.79+0^{b}
T2	64+0.58^{d}	62.11+3.76^{b}	59.81+2.32^{b}	8.14+0.006^{d}
T3	70+0^{c}	68.35+1.65^{a}	65.34+1.75^{a}	10.47+0.006^{c}
T4	64.33+0.33^{d}	62.22+10.28^{b}	53.33+0.17^{d}	10.47+0^{c}
T5	74+0.58^{a}	70.97+4.20^{a}	52.83+0.47^{d}	12.79+0^{b}

Table 6. Sensory characteristics of cooked chicken burger formulated with flaxseed flour and *Moringa oleifera* leaf.

Parameter	T4	T3	T2	T1	Control	T5
Flavor	2.28±0.346	2.47±0.342	2.71±0.34	3.66±0.354	4±0.281	1.857±0.303
Taste	1.476±0.148	1.76±0.238	2.19±0.235	3.52±0.289	4.19±0.281	1.381±0.128
Color	1.666±0.232	1.761±0.247	2.57±0.320	3.57±0.305	4.42±0.162	1.333±0.1436
Texture	1.571±0.189	1.666±0.242	2.66±0.326	3.28±0.324	3.76±0.247	1.666±0.221
Juiciness	1.428±0.176	1.381±0.188	1.80±0.272	2.42±0.28	3.33±0.326	1.333±0.173
Tenderness	1.285±0.140	1.523±0.224	2.38±0.304	3.28±0.331	3.5±0.305	1.238±0.117
Overall acceptance	1.761±0.257	1.952±0.280	2.38±0.262	3.7±0.33	4.14±0.295	1.571±0.202

Fat retention was significantly ($P \leq 0.05$) high in T1 (at 20% flaxseed flour +0% *Moringa olifera* leaf powder) , T2 (at15% flaxseed flour +5% *Moringa olifera* leaf powder and T3) at 10% flaxseed flour +10% Moringa olifera leaf powder) when compared with control samples. The increase in fat retention may be due to the fact that the swelling of the starch and fibre as well as the fat absorbed by the fibre may interact with the protein of the ground meat matrix to prevent migration of fat from the product (Alakali et al., 2010).

The shrinkage was measured by difference between two diameters of burger before and after cooking. Moreover, it can be considered as one of important quality attributes measurements. From these results, it could be observed that the control sample showed significant increase in

shrinkage compared with other treatments. Similar results were obtained with Darwish et al., (2012); Alakali et al., (2010); Al-Juhaimi et al., (2016) Shrinkage in patties during heating is caused by muscle protein denaturation and partly from the evaporation of water and drainage of melted fat and juices (Alakali et al., 2010). Treatment T2 with 5% MLF + 15% FS had the highest dimension shrinkage when compared with other treatments.

Sensory characteristics of cooked chicken burger formulated with flaxseed flour and Moringa oleifera leaf powder: The mean sensory scores of cooked chicken burger formulated with flaxseed flour and Moringa olifera leaf powder are shown in Table (6). It was observed that all sensory attributes evaluated decreased as MLF level increased and FS flour levels decreased in chicken burger formulation. The sensory attributes (taste, flavor, juiciness, tenderness, texture, color and overall acceptability) of the cooked chicken burger were significantly (P≤ 0.05) decreased in T2, T3, T4 and T5 treatments compared to control samples. Similar results were obtained with Pelser et al. (2007) who reported that incorporation of FS flour into meat products had an adverse effect on sensory attributes. Al-Juhaimi et al. (2015) obtained that the sensory attributes (appearance, juiciness, flavour, taste, tenderness and overall acceptability) of the cooked patties decreased with increasing MSF levels (M. oleifera seed flour). Treatment T1 with 0% MLF + 20% FS had the highest sensory score when compared with other treatments.

CONCLUSION

In the present study, the effect of *Moringa oleifera* and flaxseed combinations on physicochemical and sensory characteristics of chicken burger was investigated. The study concluded that a combination of FS and ML can be utilized as novel fixings to create chicken burger with high nutritive value and organoleptic properties in addition to their health benefits.

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Efficacy of Blanching and Infrared Dehydration on Phytochemical and Antioxidant Properties of the Dried *Vernonia amygdalina* Bitter Leaf Tea

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ABSTRACT

Vernonia amygdalina is a versatile plant that possesses several prophylactic and therapeutic potential with significant free radical scavenging and antioxidant activities. It's necessary to dry fresh *Vernonia amygdalina* bitter leaf into dehydrated form to extend its shelf-life for long storage. Objective of this study penetrated on the effectiveness of various processing variables such as blanching duration; infrared drying power, temperature and air velocity on total phenolic content (TPC), flavonoid content (FC), radical scavenging assay (DPPH) and ferric reducing ability of plasma (FRAP) of dried herbal tea from *Vernonia amygdalina* leaves by the infrared irradiation. The results showed that these leaves should be blanched at 25s and then being dried at power 120W, temperature 50°C with air velocity 1.4 m/s in the infrared dryer. From this study, major phytochemical and antioxidant properties such as total phenolic, flavonoid, DPPH, FRAP in the dried *Vernonia amygdalina* leaf could be preserved and maintained. Due to the advantages of the infrared irradiation, it would be an innovative approach for blanching and drying to preserve maximum valuable phytochemical and antioxidant constituents of dried *Vernonia amygdalina* leaf.

KEY WORDS: VERNONIA AMYGDALINA, BLANCHING, DRYING, INFRARED IRRADIATION, PHENOLIC, FLAVONOID, DPPH, FRAP.

INTRODUCTION

Vernonia amygdalina commonly called bitter leaf is a perennial shrub belonging to the family Asteraceae (Ijeh and Ejike, 2011). It is a perennial shrub that is widely distributed in tropical region. It's a great source of vitamins, minerals, polyphenols, alkaloids, saponins, flavonoids and steroids (Atangwho et al., 2009). This plant contains two sesquiterpene lactones: vernolide and vernodalol (Erasto et al., 2006). It is a versatile plant that possesses several prophylactic and therapeutic potential such as antidiabetic, anthelminthic, antiplasmodial, antimicrobial, antioxidant, antianaemic, immunomodulatory, cardiovascular properties (Atangwho et al., 2010; Saliu et al., 2012; Okolie et al., 2008; Akpaso et al., 2011; Ademola et al., 2011; Abay et al., 2015; Zofou et al., 2011; Omolola et al., 2013; Adesanoye et al., 2014; Oyeyemi et al., 2015; Momoh et al., 2012; Ezeonu et al., 2016; Abdulmalik et al., 2016). It is popularly consumed as a vegetable and is used for medicinal purposes such as to cure yellow fever, dysentry, constipation, malaria and stomach ache (Adegbite et al., 2009; Ebong et al., 2008, Suleiman et al., 2018).

In dehydration, the water content is decreased to a certain level where microbial proliferation will not happen while preserving the highest proximate composition. Medicinal and aromatic herbs are mostly maintained by air drying.

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Dehydrated products can be kept for a long storage and can be easily blended, powdered or packed for specific application or for deeper processing in the food or pharmaceutical purposes (Albert and Joachim, 2007). Infrared drying reveals several advantages compared to traditional drying. Infrared heating is faster than convective drying (Nowak & Lewicki, 2005). Infrared energy is transferred from the heating element to the sample, heating the material more rapidly and uniformly. Much more moisture emits from the irradiated surface and drying duration is shortened (Nowak & Lewicki, 2004). The leaf drying technique involves reducing moisture content of leaves to a point at which biochemical changes are limited while maintaining cell structure, pigment content and overall appearance (Singh and Dhaduk, 2005, Zaharaddeen and Samuel, 2019).

There are several notable studies mentioned to drying of *Vernonia amygdalina* species. The drying behavior of *Vernonia amygdalina* leaves was investigated using open sun and shade drying. Open sun drying resulted in severe deformity of the leaf morphology which may lead to degradation of the phytochemicals (Oluwaseun et al., 2018). The effect of air, sun, oven and solar drying methods on the organic and dietary elemental composition of *Vernonia amygdalina* leaves was evaluated. Drying improved the concentration of both organic and dietary elemental components (Zaharaddeen and Samuel, 2019). Objective of this present study focused on the effectiveness of blanching, infrared drying power, temperature and velocity on total phenolic, flavonoid, DPPH, FRAP of dried herbal tea from *Vernonia amygdalina* leaves under the infrared irradiation.

MATERIAL AND METHODS

Vernonia amygdalina leaves were collected from Soc Trang province, Vietnam. After collecting, they must be kept in cool and dry cotton box, conveyed to laboratory for experiments. They were subjected to the blanching and infrared dehydration under different conditions. All standards and reagents such as Folin-Ciocalteu reagent, Na_2CO_3, gallic acid, $Al(NO_3)3$, potassium acetate, DPPH, methanol, ethanol, acetate buffer, 2,4,6- tripyridyl-s-triazine, HCl , $FeCl_3.6H_2O$ were analytical grade and purchased from Sigma-Aldrich. Lab utensils and equipments included weight balance, blender, infrared dryer, spectrophotometer.

Effect of blanching duration (s) to phytochemical and antioxidant properties of *Vernonia amygdalina* leaf: Raw *Vernonia amygdalina* leaves were blanched at 100oC in different duration (15, 20, 25, 30, 35s). After being blanching, these blanched leaves would be would be dried by infrared dryer with drying power 40W at temperature 40oC with air velocity 0.8 m/s. The dried samples were analyzed the total phenolic (mg GAE/100 g), flavonoid (mg QE/100 g), DPPH (%) and FRAP (mM TE/100g).

Effect of infrared drying power (W) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf: After finding the optimal blanching condition, these blanched leaves would be dried by infrared dryer under different powers (40, 80, 120, 160, 200 W) at temperature 40oC with air velocity 0.8 m/s. After this experiment, the dried leaves would be analyzed the total phenolic (mg GAE/100 g), flavonoid (mg QE/100 g), DPPH (%) and FRAP (mM TE/100g).

Effect of infrared drying temperature (°C) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf: By selecting the optimal steaming time and drying power, these blanched leaves would be dried by infrared dryer under power 120 W at different temperature (40, 45, 50, 55, 60°C) with air velocity 0.8 m/s. After this experiment, the dried leaves would be analyzed the total phenolic (mg GAE/100 g), flavonoid (mg QE/100 g), DPPH (%) and FRAP (mM TE/100g).

Effect of infrared drying air velocity (m/s) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf: By selecting the optimal steaming time, drying power and drying temperature, these blanched leaves would be dried by infrared dryer under power 120 W at temperature 50oC with different air velocity values (0.8, 1.0, 1.2, 1.4, 1.6 m/s). After this experiment, the dried leaves would be analyzed the total phenolic (mg GAE/100 g), flavonoid (mg QE/100 g), DPPH (%) and FRAP (mM TE/100g).

Phytochemical and antioxidant estimation: Total phenolic content (mg GAE/100g) was evaluated using Folin–Ciocalteu assay (Nizar et al., 2014). Total flavonoid content (mg QE/100g) was evaluated by the aluminium calorimetric method (Formagio et al., 2015). The antioxidant activity was evaluated using DPPH (%) radical scavenging assay which was described by Huang et al. (2005). FRAP (mM TE/100g). FRAP (mM TE/100g) were performed according to Ivanov et al. (2014).

Statistical analysis: The experiments were run in triplicate with three different lots of samples. The data were presented as mean±standard deviation. Statistical analysis was performed by the Statgraphics Centurion version XVI.

RESULTS AND DISCUSSION

Effect of blanching time (s) to the phytochemical and antioxidant properties of *Vernonia amygdalina* leaf: Blanching is an important step before drying of herbal materials to inactivate enzymes that cause browning and loss of bioactive constituents. Raw *Vernonia amygdalina* leaves were blanched by hot water at 100°C in different durations (15, 20, 25, 30, 35s). After being blanching, these blanched leaves would be would be dried by infrared dryer with drying power 40W at temperature 40°C in air velocity 0.8 m/s. Results are presented in table 1.

It's clearly noticed that 25s in blanching was adequate to maintain the highest amount of total phenolic,

flavonoid, DPPH and FRAP. So this value was selected for further experiments. Omede et al. (2018) evaluated the antioxidant activity and cytotoxic properties of *Vernonia amygdalina*. They confirmed that the extracts possessed very low cytotoxicity (IC50 = 1.83 mg/ml), high total phenolic content (158.8 mg GAE/100g), total flavonoid (85.7 mg QE/100g).

Table 1. Effect of blanching time (s) to the phytochemical and antioxidant properties of *Vernonia amygdalina* leaf

Blanching duration (s)	15	20	25	30	35
Total phenolic (mg GAE/100g)	24.51±0.02^{b}	26.30±0.01^{ab}	29.47±0.02^{a}	22.79±0.03^{bc}	20.05±0.01^{c}
Total flavonoid (mg QE/100g)	10.73±0.01^{b}	12.64±0.00^{ab}	14.55±0.03^{a}	9.17±0.01^{bc}	7.32±0.00^{c}
DPPH (%)	31.50±0.00^{c}	33.79±0.03^{b}	36.04±0.01^{a}	34.83±0.00^{ab}	32.65±0.02^{bc}
FRAP (mM TE/100g)	11.74±0.02^{c}	13.01±0.01^{b}	15.48±0.00^{a}	14.84±0.02^{ab}	12.36±0.03^{bc}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α= 5%).

Effect of infrared drying power (W) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf: Drying can lead to considerable loss of the available bioactive components due to thermal degradation depending on the drying method and temperature conditions (Naomi et al., 2018).

They proved that total phenolic content, antioxidant capacity were significantly affected by drying method and drying temperature. By finding the optimal blanching condition, these blanched leaves would be dried by infrared dryer under different powers (40, 80, 120, 160, 200 W) at temperature 40°C with air velocity 0.8 m/s. Our results were presented in table 2. It's clearly noted that the optimal infrared drying power should be 160 W to preserve the best phytochemical and antioxidant attributes of dried *Vernonia amygdalina* leaf. So this value was selected for further experiments.

Table 2. Effect of infrared drying power (W) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf

Drying power (W)	40	80	120	160	200
Total phenolic (mg GAE/100g)	29.47±0.02^{c}	33.49±0.03^{bc}	38.64±0.01^{a}	36.12±0.00^{ab}	34.41±0.03^{b}
Total flavonoid (mg QE/100g)	14.55±0.03^{c}	15.74±0.01^{bc}	17.63±0.03^{a}	17.05±0.02^{ab}	16.54±0.00^{b}
DPPH (%)	36.04±0.01^{bc}	38.61±0.00^{ab}	40.82±0.02^{a}	37.53±0.01^{b}	33.71±0.02^{c}
FRAP (mM TE/100g)	15.48±0.00^{c}	17.01±0.02^{b}	19.26±0.00^{a}	18.95±0.03^{ab}	16.58±0.01^{bc}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α= 5%).

Effect of infrared drying temperature (°C) the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf: Infrared irradiation has potential for drying herbs because it is gentle and shortens the processing duration. The leaf drying is an important post-harvest strategy for improving product quality as well as providing added value. By selecting the optimal steaming time and drying power, these blanched leaves would be dried by infrared dryer under power 120W at different temperature (40, 45, 50, 55, 60°C) with air velocity 0.8 m/s.

Our results are shown in table 3. The optimal drying temperature was recorded at 50°C to preserve the best phytochemical and antioxidant attributes of dried *Vernonia amygdalina* leaf so we choose this value for further experiments. In one report, Oluwaseun et al. (2018) proved that shade drying was the better way of drying V. amygdalina leaves to preserve the nutrients compared to open sun drying.

Table 3. Effect of infrared drying temperature (oC) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf

Drying temperature (oC)	40	45	50	55	60
Total phenolic (mg GAE/100g)	38.64±0.01^{c}	39.14±0.03^{bc}	40.53±0.02^{a}	40.09±0.03^{ab}	39.64±0.00^{b}
Total flavonoid (mg QE/100g)	17.63±0.03^{c}	18.25±0.01^{bc}	19.78±0.00^{a}	19.33±0.01^{ab}	18.96±0.03^{b}
DPPH (%)	40.82±0.02^{c}	41.98±0.00^{ab}	42.56±0.01^{a}	41.32±0.02^{b}	41.07±0.00^{bc}
FRAP (mM TE/100g)	19.26±0.00^{c}	20.76±0.02^{bc}	21.83±0.03^{a}	21.35±0.00^{ab}	21.01±0.01^{b}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Effect of infrared drying air velocity (m/s) the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf: By selecting the optimal steaming time, drying power and drying temperature, these blanched leaves would be dried by infrared dryer under power 12 W at temperature 50oC with different air velocity values (0.8, 1.0, 1.2, 1.4, 1.6 m/s). Our results are shown in table 4.

Table 4. Effect of air drying velocity (m/s) to the phytochemical and antioxidant properties of dried *Vernonia amygdalina* leaf.

Air drying velocity (m/s)	0.8	1.0	1.2	1.4	1.6
Total phenolic (mg GAE/100g)	40.53±0.02^{b}	40.98±0.01^{ab}	41.25±0.03^{ab}	41.39±0.00^{a}	41.43±0.01^{a}
Total flavonoid (mg QE/100g)	19.78±0.00^{b}	20.03±0.03^{ab}	20.27±0.01^{ab}	20.88±0.02^{a}	20.91±0.03^{a}
DPPH (%)	42.56±0.01^{b}	42.88±0.02^{ab}	42.97±0.00^{ab}	43.31±0.03^{a}	43.36±0.02^{a}
FRAP (mM TE/100g)	21.83±0.03^{b}	22.01±0.00^{ab}	22.34±0.02^{ab}	22.65±0.00^{a}	22.69±0.01^{a}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%)

The optimal air velocity was recorded at 1.4 m/s to preserve the best phytochemical and antioxidant attributes of dried *Vernonia amygdalina* leaf so we choose this value for application. In one study, Zaharaddeen and Samuel (2019) proved that oven–solar drying method could be useful in preserving *Vernonia amygdalina* leaves in a more hygienic way and ensure its all-the-year round availability and possibly elimination of most nutrient deficiencies.

CONCLUSION

V. amygdalina contains numerous phytochemical constituents associating with its potent pharmacological properties to treat various ailments. We have successfully examined different technical variables influencing to the blanching and drying process of *Vernonia amygdalina* leaf. Due to low moisture content, these dried leaves can be kept in ambient environment for longer periods without losing their appearance and pharmaceutical value. *V. amygdalina* would be a safe potential source of natural antioxidant agent as a functional food.

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Transmission Electron Microscopy Study of Multidrug Resistant of *Enterococcus faecalis* and amplification of vanA Gene in Clinical Samples

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ABSTRACT

Vancomycin resistant *Enterococcus faecal* is a concern for both public health and medicine, as its importance is associated with serious multidrug resistant. It is an opportunistic pathogen which represents one of the agents of nosocomial infection in hospitalized patients. The aim of the present study was to determine the cell wall thickness by transmission electron microscopy and uncovering of resistant gene responsible gene for the vancomycin resistance *E. faecalis* strains isolated by clinical samples from Gulbarga region. Isolation and identification of *E. faecalis* of clinical isolates were done by using standard culturing and screening protocol. Antibiotic susceptibility and MIC were determined by using disc diffusion and broth dilution method. The thickness of cell wall of the stains was studied by using TEM and vanA gene was detected by Polymerase Chain reaction. In our study among 76 isolates of *E. faecalis*, 12 strains showed high resistance to gentamycin and vancomycin antibiotics. TEM-analysis showed cell wall and increased sepate as compared to the normal *E. faecalis* 122 strain and standard culture *E. faecalis* NCIM 5625. The VREF 122 strain showed amplified product size of 352 pb of vanA gene. The study on TEM provides better evaluation in cell wall thickness treated with vancomycin antibiotic and PCR for the detection of resistant gene. These findings indicated the cell wall thickness is a characteristic phenotype of *E. faecalis* exposed to vancomycin antibiotic and PCR reveals the presence of the vanA gene. It is therefore believed that cell wall thickness and detection of resistance gene plays an important role in mechanism of action of Vancomycin antibiotic. Further these tools may find use in markup application employing detection of resistance pattern in the bacteria.

KEY WORDS: E.FAECALIS, TEM, VANA GENE, MDR, VRE, CELL WALL THICKNESS.

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INTRODUCTION

Enterococci are commensal organism that acts as opportunistic pathogens. Currently, they have been studied to be among leading pathogens of nosocomial infections, and thus they are a major international health burden (Dulber et al., 2020).Among the enterococci, *Enterococcus faecalis* cause 80-90 % of infections and it is a gram-positive commensal member of the gut microbiota of wide range organisms. With the advent of antibiotic therapy, it has emerged as multidrug resistant, hospital acquired pathogen (Daria et al., 2013). It majorly causes in humans such as bacteremia, endocarditis, intra-abdominal, pelvic infections and urinary tract infections. However, inappropriate vancomycin use has resulted in the emergence of Vancomycin resistant enterococci (VRE) and capably of transferring the resistance gene to another organism. VRE infection can be acquired through colonization with VRE or hospital environmental (Jatapat et al., 2019). Enterococci gladly accrue mutations and exogenous genes that confer additional resistance. They develop resistance to vancomycin by exchange of genetic material among themselves and or with another genera. The enterococci may acquire resistance through van associated genetic elements like *vanA, vanB, vanD, vanE, vanG, VanL* of which *vanA* and *vanB* are the most prevalent genotypes in clinical isolates (Addisu et al., 2020).

In Asian countries the prevalence was decreased may due to recent emergence of this resistance in this continent and only a handful of studies to document. The prevalence of VRE in India has been reported the highest was in New Delhi with 23%, Chandigarh 8% and lowest in Mumbai with 5.5% respectively and all of *vanB* phenotype study reported by Manimala et al., (2019). Transmission electron microscopy will insightful impact on understanding of bacteria and other microbial populations, it provides the 1000 fold improvement in resolution and allows to find the minor changes in topological and structure of bio-membrane organelles. (Shruthi and Vivek 2017).It is important to study the prevalence, pattern of resistance especially in this region of South India which has very few reports on *Enterococcus faecalis*. The present objective of the study to better understand the antimicrobial action and changes in cell wall thickness induced by vancomycin in *E. faecalis*.

MATERIAL AND METHODS

Isolation of *Enterococcus faecalis*: The samples were examined for the presence of pathogenic. In the present study bile esculin azide agar was used for primary isolation of *E. faecalis*. Microscopic observation was done for confirmation and subculture again 3 to 4 times to isolate pure culture of *E. faecalis*. The single isolated colony was inoculated in BHI broth and incubated at 37°C for 18 hrs, glycerol (30%) were used for preparation of stock culture and stored in -80 °C for further use.

Antimicrobial Susceptibility testing: The standard disk diffusion method was used to perform antimicrobial susceptibility testing on Muller Hinton agar (Hi-Media, India) as recommended by the Clinical and Laboratory Standards Institute (CLSI 2007). The most commonly used antibiotics used for the tests were vancomycin, ampicillin, oxacillin, rifamycin, ciprofloxacin, tobramycin, gentamycin, teicoplanin and streptomycin.

Determination of minimal inhibitory concentration (MIC's) of Vancomycin and Gentamycin by broth dilution method: MIC for antibiotic (i.e, Gentamycin and Vancomycin) concentration ranging from 6-256 µg/ml and 512 to 1026µg/ml respectively were prepared with BHI broth and used to test each isolate and final concentration of each antibiotic were 10 mg/ml.

Transmission Electron Microscopy: TEM was performed for the morphological characterization of the cell wall ultra-structure of GREF and VREF isolates. The protocol for the preparation and examination was followed as described earlier (Hanaki et al., 1998). The overnight culture was centrifuge at 8000 rpm for 10 mins 4°C, the supernatant fluids were discarded, and the cell pellets were washed twice with 50 mM potassium phosphate buffer (pH 7.0). Bacteria were then fixed in 3 % glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.2). Then followed bacteria were fixed with 3% glutaraldehyde for overnight at 4°C. For high contrast amplification, the bacterial cells were treated with 2 % uranyl acetate in 95 % alcohol for 1 h at 20° C in the dark. Infiltration is done with help of araldite propylene oxide (1:1) solution for five times. Cells were subjected to dehydration with 70 % ethanol for 18 hrs in acrylic resin. The ultra-microtome was used to prepare ultra-thin section, finally stained with lead citrate and uranyl acetate and examined under Transmission electron (Techn G2 Spirit TEM, at 80 KV).

Plasmid DNA Analysis: The plasmid isolation kit (Bangalore gene pvt.ltd) were used for the extraction of Plasmid DNA from vancomycin resistant isolates by user manual of kit, further it was analyzed in 0.8% agarose gel electrophoresis containing 5 µg/mL of ethidium bromide at 3.5 V/ cm for 4 h in a Tris EDTA buffer (TAE). Molecular markers were used as a size reference for molecular determinations.

vanA Gene Amplification: Plasmid DNA from vancomycin resistant enterococci isolated by the plasmid isolation kit (Bangalore gene pvt. ltd) were subjected to amplification assays employing the oligonucleotide forward primer (5'-GGGAAAACGACAATTGC-3') and reverse primer (5'-GTACAATGCGGC CGTTA-3') and amplification conditions as described by (Dutka et al.,1995). The PCR reaction consisted of Initial denaturation at 94 ˚C for 10 mins, followed by 35 cycles of denaturation to 94˚C for 30 secs, annealing to 47 ˚C for 45 secs, then extension to 72˚C for 30 secs. Followed by final extension at 72 ˚C for 10 mins. The amplified products were resolved by electrophoresis on a 1% Agarose–TAE gel containing gel red.

RESULTS AND DISCUSSION

Bacterial Isolates and Antimicrobial Susceptibility Test: In the present study, strains of *E. faecalis* were isolated from clinical samples A total of 250 samples were collected, among them 122 have showed positive for different clinical isolates. The E. faecalis 76 (62.29 %) was the predominant isolates from urine, pus, CSF and blood samples. The *E. faecalis* isolates were Gram positive. The biochemical characteristics for the all isolates were positive for tellurite reduction and arginine hydrolysis, arabinose, raffinose and mannitol expect for catalase sorbitol and lactose. The results of the susceptibility tests are carried out by disc diffusion method for 76 *E. faecalis* strains showed resistant to the different antibiotic like vancomycin (77.63%), gentamycin (64.47%) and oxacillin (55.26%) antibiotics, and were multi drug resistant. The isolates were found sensitive to rifamycin (61.84%), teicoplanin (55.26%) streptomycin 52.63%) and tobramycin (51.13%).

Figure 1: Transmission electron micrographs of *E. faecalis* (A) Control Isolate *E. faecalis*
EF122 (B) Control of *E. faecalis* EF122 Septum; (C) NCIM 5205 E. faecalis Std
Strain; (D) NCIM 5205 *E. faecalis* Std. Strain septum magnification at X68, 000.

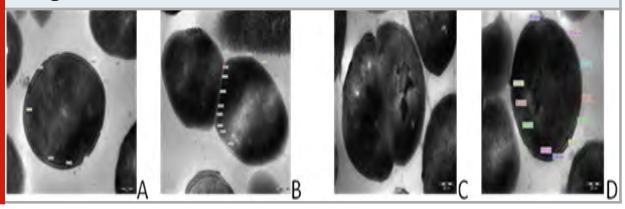
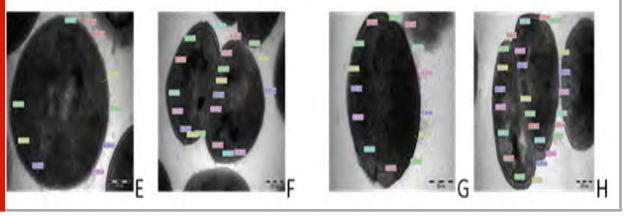


Figure 2. Transmission electron micrographs of *E. faecalis* isolates (E) Gentamycin treated
E. faecalis EF122 isolate; (F) Gentamycin treated *E. faecalis* EF 122 isolate of
Septum;(G) Vancomycin treated *E. faecalis* EF 122 isolate;(H) Vancomycin
Treated *E. faecalis* EF122 isolate septum magnification at X68,000



Determination of MIC's in *E. faecalis* isolates: MIC's for gentamycin among 76 isolated 12 *E. faecalis* high resistance to gentamycin and Vancomycin among them 5 isolates showed ≥1024 µg/ml and 5 isolates had MIC of ≥512 µg/ml and 2 strains showed 256 µg/ml. The vancomycin MIC for 8 isolates showed ≥64 µg/ml and 4 isolates had ≥ 128 µg/ml of the total 12 isolates were tested as shown in the Table.1.

Transmission Electron microscopy: TEM was employed to examine the cell morphology (especially cell wall thickness) of *E. faecalis* in in-vitro using gentamycin and vancomycin. Using broth dilution method, *E. faecalis* (GREF 122 and VREF 122) was adapted during MIC study and further sub cultured and stored at -80° C (expressed high MICs, ≥1024 µg/ml and 128 µg/ml). To ensure the resistance mechanism of *E. faecalis* to gentamycin and vancomycin, the TEM was carried out along with control isolates (EF122 and NCIM 5025). The isolates showing baseline resistance of 16 µg/ml MIC were subjected to chemical fixation prior to examination.

Figure 3: PCR amplification of vanA gene Lane M- 100bp ladder and Lane-1 amplified Product vanA gene of *E. faecalis* EF122

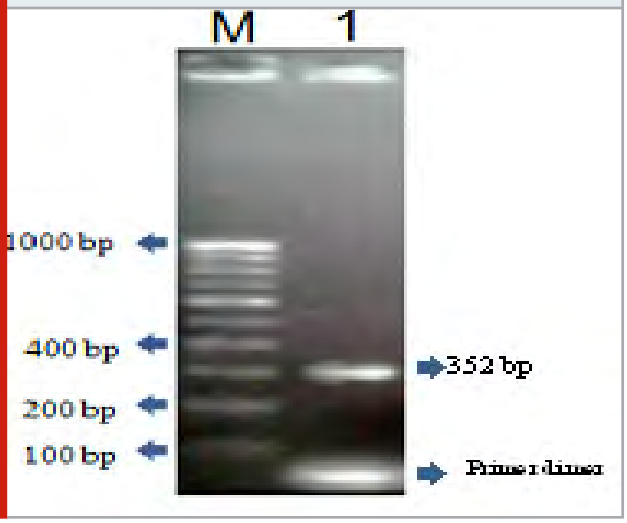


Table 1. MICs determination of Gentamycin and Vancomycin resistance of *E. faecalis* Isolates

Isolates	Sample	MIC µg/ml	
		Gentamycin	Vancomycin
121	Urine	1024	128
122	Urine	1024	128
123	Urine	1024	128
124	Urine	1024	64
125	Urine	1024	64
136	Blood	512	64
137	Blood	512	64
138	Blood	512	64
139	Blood	512	64
140	Blood	512	64
141	CSF	256	128
142	Pus	256	64

TEM photographs showed that there was significant alteration in the thickness of the bacterial cell wall. The control strain EF 122 showed the cell wall thickness of 30.598 nm and septum of 18.902 nm with X68,000

magnification as shown in Fig.1 a & b. The standard strain NCIM *E. faecalis* 5025 showed 30.832 nm cell wall thickness and septum of 24.358 nm with X68, 000 magnifications as shown in Fig.1 c & d. The isolates when exposed to gentamycin the cell wall and septum thickness was increased in size of 40.164 nm and 35.770 nm respectively at magnification at X68, 000 as show in Fig. 2 e & f. Similarly, the cell wall and septum thickness was increased higher in size of 47.842 nm and 46.358 nm respectively for vancomycin antibiotic as shown in Fig. 2 g & h.

Plasmid DNA analysis and *vanA* gene amplification: Figure 3. Reports the plasmid profile of human clinical isolates. The two isolates 121 and 122 are independently of their origin had more than one plasmid with different molecular weight. Among the observed plasmid, those ranging from 2 strains, more than one plasmid species were detected in isolates. Among the observed plasmids, those ranging approximately from 21 and 19 kbp in molecular size. (Not data shown) shows an example of amplification of the 352 pb fragment in plasmid DNA of isolates, confirming the affiliation to the *vanA*

Table 2. Averaged means values of Cell wall thickness of the isolates

Name of the isolates	Measurement of thickness magnification at X 68,000 nm	
	Cell wall	Septum
Control Strain EF 122 Standard Culture	30.598 nm	18.902 nm
E. faecalis NCIM 5025	30.832 nm	24.358 nm
Antibiotic treated EF 122 (Gentamycin) Strain	40.164 nm	35.770 nm
Antibiotic treated EF 122 (Vancomycin) Strain	47.842 nm	46.358 nm

gene cluster. This result also demonstrates the extra chromosomal location of the glycopeptide resistance determinants. The amplified PCR product was sequenced and submitted to NCBI (Acc .No JN791694).

Drug resistant infectious provides opportunities to address the associated clinical and public health burden on individuals, health systems, and society, (Olaniyi et al., 2020). Worldwide there is a trend in increasing of vancomycin resistant enterococci ever since first described in 1987, although the epidemiology of these microorganism varies widely in different geographical areas (Hossein and Mohammad 2014). *E. faecalis* has recently evolved from a generally a virulent commensal into an multi-drug resistant healthcare-associated pathogen causing difficult-to-treat infections. Therefore, studies of *E. faecalis* resistance have increased. The most species distribution of the enterococcus species was *E. faecalis* with (81.72%) isolated, followed by *E. faecium* (12.9%) as reported by Jahnabi et al., 2016. In recent study they have been reported has (64%) of *E. faecalis* and *E. faecium* (36%) in Chennai, India (Alexander et al., 2020).

In our studies *E. faecalis* occurrence was (62.29%) the difference may be due to geographical variation. Vancomycin resistance was high with numbers of screened isolates were less 28/31 (90.62%) reported in North Indian tertiary care hospital (Hibs et al., 2020). In the year 2016 a study showed high rate (35.3%) of VRE carriage as compared to studies that have estimated comparatively low VRE colonization on admission to the ICU from Europe (2.7%), US (12.3%), South America (7%) and other Asian countries (5.3%) (Prakhar et al., 2016). In our study, about 77.64 % of *E. faecalis* strains showed high resistant to vancomycin by disk diffusion test and MIC of 12 strains as showed ≥ 64 to ≥128 µg/ml for vancomycin respectively. The last therapeutic resort for enterococci was vancomycin.

Transmission electron microscopy has been invaluable tool in imaging bacteria and their components. The first report to show cell wall thickening was in S. aureus following treatment with acriflavine. Beta-lactam antibiotics which inhibit cell wall synthesis cause marked thickening of the cross wall in Staphyloccocci. MRSA has showed that vancomycin is affinity trapped inside the peptidoglycan layers by false targets, resulting in peripheral wall thickening (Mako and Jun 2009). In present study the isolate vancomycin resistant *E. faecalis* EF122 showed lesser thickness of cell wall and septum compared standard strain NCIM E. feacalis 5025 when grown in the medium without vancomycin. In contrast, when the isolate exposed to 16 µg/ml of vancomycin and gentamycin increased thickness of cell wall was 9.564 nm and 16.566 nm against gentamycin and vancomycin, whereas in case of septum 26.868 nm and 27.456 nm with gentamycin and vancomycin respectively.

In our study PCR proved helpful in detecting the van genotypes present in this geographic region. The study reveals an expression of *vanA* gene in the *E. faecalis* strain with 352bp fragment in the plasmid DNA and it confirms isolates the affiliation to the vanA gene cluster and the extra chromosomal location of the glycopeptide-resistance determinants. The *vanA* type resistance is dominant in the United States and Europe whereas *vanB* type resistance is more frequent in Australia and Southeast Asia, (Nerfis and Baris 2020). However, VRE strains carrying vanB or both vanA/vanB have reported in various countries (Coombs et al., 2014, Marcade et al., 2014, Papagiannitsis et al., 2017). In India the most of strains of VRE has showed the vanA genotype (Hiba et al., 2020). In our study, the increase in the rate of prevalence of *E. faecalis* and emergence of multidrug resistance among them (Ajay et al., 2012), highlights the significance of rapid and accurate in identification of enterococci to the species level for imitating appropriate therapeutic regimen, and reemphasizes the importance of the implementation of appropriate infection control measures to limit the nosocomial spread of these unusual species in any nosocomial.

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Conflict of Interest: None

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Parasitological Communication

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Current Status and Impact of Animal Parasitic Nematodes : A Review

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ABSTRACT

Nematodes are the most abundant metazoans on this planet that have been reported from all terrains of all the continents. They are free-living as well as parasitic, living in both plants and animals. Parasitic nematodes are most important because they severely affect their host thereby causing significant economic loss. The animal parasitic nematodes cause several debilitating diseases in human, livestock and other domestic animals. Among various other features, nematodes are most diverse group of animals in terms of their size and life-span. Some of the diseases caused by parasitic nematodes in humans have become priority of global health. Large scale sequencing of the genomes from parasitic nematodes itself highlights their impact on human survival. In order to better understand the situation, in-depth knowledge about the parasite, its impact on socio-economic conditions is must. Data on these accounts help in deciding the course of action, area to focus and making informed policy decisions. New approaches have undoubtedly helped human kind with various treatment option and better socio-economic status. There are several important parasitic nematodes with reference to human and veterinary importance. This review covers the impact of the common parasitic nematode infecting humans as well as animals and highlights the current issues and prospects associated with the management of these nematodes.

KEY WORDS: ASCARIS; IMPACT; NEMATODES; PARASITES; WHO.

INTRODUCTION

The word 'helminth' is a general term, which is often used to convey one or the other form of parasitic infections. In academic and research world, it is a broad term encompassing all known flatworms and roundworms. Helminths are metazoan animals with multi-cellular arrangement and a body which is like a tube within tube. Outer tube is a tough cuticular skin and the inner tube contains all the body parts including digestive and reproductive systems. These animals exhibit bilateral symmetry, triploblastic in terms of muscular arrangement and important pseudocoelomatic creatures of the nature (Okwa, 2020). While majority of helminths are free-living in aquatic and terrestrial environment, few are parasites in human and plants. It is the parasitic forms which have received much attention due to the diseases they cause in both humans as well as in plants (Elton, 2020, Combes, 2020).

Helminths are very special compared to other parasites in nature (Rapin & Harris, 2018). Their development is quite slow compared to other infectious pathogens. Diseases caused by helminths have slow onset but are chronic in nature (Mutapi et al., 2017). Although mostly go as asymptomatic, helminths infection cause severe economic damage worldwide and are associated with

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high level of morbidity and mortality (Krolewiecki & Nutman, 2019). All human parasitic nematodes have similar life cycle with certain notable differences (Ancell & Pires-daSilva, 2017, Jex, et al., 2019). They demonstrate a well developed sexual dimorphism. Females of the parasitic nematode produces large number of eggs that after hatching pass through four larval stages and turn into an adult (Chaudhuri et al., 2011). Understanding the life-cycle of animal parasitic nematode is essential to identify the vulnerable stages which can be targeted for its management. Common route of transmission adopted by nematode parasite includes faecal-oral transmission, transdermal transmission, vector-borne transmission and predator-prey transmission (Furtado et al., 2020).

Infection by parasitic nematodes is a common problem throughout the world. Their enormous distribution and infections in human population can be understood by a fact that in year 1946, it was estimated that a population of 2.2 billion at that time had 2.3 billion nematode infections. Each human being was infected by more than one nematode (Becker et al., 2018). Their impact on the lives of human and animals is very drastic and frightening. Nematode infections are a cause of serious problem in the developing world where intestinal infections are the most frequent ones. Common intestinal nematodes from the developing world are *Ascaris lumbricoides, Trichuris trichiura* and *Strongyloides stercoralis* (Table-1). Important nematodes of veterinary importance are *Ostertagia ostertagi, Cooperia oncophora, Teladorsagia circumcincta, Haemonchus contortus, Dictyocaulus viviparous*. Details of each of these nematodes are listed (Table-2).

From human perspective, these three nematodes account for three-quarter of all infections (Freeman et al., 2019). Global atlas on nematode infection is compiled based on the findings of several research groups. Throughout the world, more than half of the population is affected by nematode infections. Even in the presence of modern medical aids, incidences of nematode infections are continuously increasing mostly from the developing world (Van Den Hoogen et al., 2019). A number of factors are responsible for this increase, which includes lack of awareness about proper sanitation and health related issues. Treatment of the nematode infection depends mostly on chemical based medicines (Werkman et al., 2020). Recently, the progresses in research have led to discoveries of plant based antihelmintic treatments (Liu et al., 2020, Zajícková et al., 2020).

In order to make informed policy decisions and plan better management strategies, detailed and meaningful data is required. This review presents the insight and highlights the impact of the nematode infection on human and animals. Progress in the field of parasitic nematodes has not seen much pace compared to other fields. Major reason for this slow growth had been the biology of these animals. These animals live deep inside their host, are bio-trophic in nature, have long life-cycles, are difficult to culture in laboratory outside their host etc. Through this review an attempt has been made to summarize the important parasitic helminths which are responsible for significant morbidity in both humans as well as animals. In order to summarize the details, we have looked in to various publication, looked in to metadata presented, searched important search engines and presented the updated details.

Reason Behind The Ignorance: There are around 342 species of nematode that infect human beings (Laurimaa et al., 2016). Nematode infections have increased with increasing human population globally (Sorobetea et al., 2018). Published data suggests that there are significant numbers of infections reported worldwide. Out of the 3500 million infections worldwide, there are 450 million individual who require serious medical attention. As per recorded data, more than 125000 deaths occur every year due to nematode infections, particularly by *Ancyclostoma* (Coulibaly et al., 2019). The reason behind ignorance of nematode infection is primarily due to their asymptomatic and non fatal nature compared to the infections cause by protozoan parasites especially malaria, which receives so much public attention and huge research funding (Rückerl, 2020). Most common symptoms associated with parasitic nematodes infection in humans include abdominal pain, diarrhoea, malnutrition, and anaemia (Tamarozzi et al., 2019). In certain case of *Trichuris* infection, cognitive function is impaired to some extent, due to secondary infections by opportunistic pathogens etc. Details about the mechanism of infection, adverse effect of infection and the economical impact due to these infections can be gained from several excellent reviews (Jourdan et al., 2018, Wright et al., 2018, Ramlal et al., 2019 Norman et al., 2020).

Problem in Management of Nematode Infections: As mentioned earlier that good level of personal hygiene, proper sanitation and health-related education is a must for creating awareness against these infections. Based on the published reports, only personal hygiene helps in reducing the rising cases of nematode infections throughout the globe (World Health Organization, 2018). There are various challenges associated with management of nematode infections. Since the problem of nematode infection is a global problem, its management becomes more complex because of varying level of environmental, social and economic factors across different countries. Scientists have pointed out that it is next to impossible to get our world free from nematode infections, but certainly we should be able to manage it. In order to control or limit the infections caused by helminths, focus must be on reducing contact based transmission of parasites as this strategy will help in reducing the risk of spreading further infections (World Health Organization, 2019).

Control Methods: The treatment and control of animal parasitic nematode infection is primarily dependent on anthelmintic drugs. Benzimidazoles group of medicines like albendazole and mebendazole, imidazothiazoles group of medicines such as levamisole and pyrantel are common chemical anthelmintic drugs recommended by

various agencies (Enejo & Suleiman, 2017; Gandasegui et al., 2020). Thiabendazole, which is structurally related to albendazole and mebendazole, is used widely for the treatment of several nematodes of cattle, horses, and sheep (Legarda-Ceballos et al., 2016). Dithiazanine is another nematode anthelmintic used in veterinary medicine; it is effective against heartworms and threadworms. Diethylcarbamazine is the drug of choice for treatment of filariasis caused by a parasitic nematode, *Wuchereria bancrofti* throughout the globe (McCarthy & Moore, 2015, Misra-Bhattacharya & Shahab, 2018).

Though, a polytherapy treatment that includes ivermectin with diethylcarbamazine or albendazole is more effective than either drug alone. Macrocyclic lactones group of medicines are an important class of anthelmintics for the control of nematode parasites and some ectoparasites in livestock, companion animals and in humans (Prichard & Geary 2019). Similarly, pyrantel pamoate is effective against *Ancyclostoma*. As a part of integrated pest management, scientists are looking beyond the reliance only on chemical based anthelmintics for treatment of parasitic nematode infections. Certain plant metabolites have been also evaluated for controlling nematode diseases of humans (Athanasiadou et al., 2007, Punetha et al., 2020). Use of plant based products for controlling livestock nematodes has also been tried. Plant based products offer alternative methods of controlling animal parasitic nematodes (Behera & Bhatnagar, 2018, Garcia-Bustos et al., 2019).

Table 1. List of important nematodes causing significant infection in humans

S.No	Name of the parasite	Common Name	Clinical symptoms	Transmission	Distribution	Number of people infected worldwide	References
1.	*Ascaris lumbricoides*	Roundworm	Abdominal pain, diarrhoea, malnutrition	Ingestion of eggs	Worldwide	807-1,121 Million	(Shah and Shahidullah, 2018)
2.	*Ancylostoma duodenale*	Hookworm	Cough, dyspnea and Hemoptysis	Contaminated soil	Worldwide	576-740 Million	(Giramkar, 2020)
3.	*Trichuris trichiura*	Whipworms	Abdominal pain, unexpected weight loss	Contaminated soil and food	Worldwide	604-795 Million	(Else. et al., 2020)
4.	*Onchocerca volvulus*	River Blindness worm	Skin and lymph node inflammation	Repeated bites by black flies	Sub-Saharan Africa	187 Million	(Otabil. et al., 2019)
5.	*Wuchereria bancrofti*	Filarial worm	Lymphedema,	Through the bite of an infectious mosquito	Africa and India	893 Million	(Zulch. et al., 2020)
6.	*Brugia malayi*	Filarial worm	Ulceration of the affected lymph node	Mosquito vector	South-east Asia	120 Million	(Liu et al, 2018)
7.	*Dracunculus medinensis*	Guinea worm	Nausea, vomiting, Blisters.	By drinking unfiltered water	Remote areas of Africa	3.5 Million	(Robert L, 2019)
8.	*Enterobius vermicularis*	Pin worm	Perianal pruritus	Ingestion of Infectious eggs	Worldwide	1.0 Billion	(Fan et al., 2019)

Socio-Ecomonic Impact of Helminths Infection: Significant morbidity and mortality takes places due to infections by helminths (Feasey et al., 2010, Gadoth, 2019). Many regions of the world, especially the developing world are facing severe problem due to the infections caused by helminths (De Rycker et al., 2018). Unhygienic living conditions and non-availability of safe drinking water is directly linked to the development and proliferation of these diseases. In essence, parasitic diseases caused by helminths are considered to be the diseases of poor and exhibit significant socio-economic impact on the society (Lindahl & Grace, 2015). The

severity of the impact can be felt through the disease burden both in human as well as in livestock. Developing countries are particularly are at the risk of this uninvited socio-economic burden due to large population, poor hygienic conditions and lack of awareness (Gizaw et al, 2020).

Significant progress has been made by agencies such as United Nations Development programme and World Health Organization in assessing the socio-economic impact of these tropical diseases (Bangert et al., 2017). With the aid from these agencies, global disease burden can be minimized either through eradication or controlling specific diseases. All such project will surely involve the cost effectiveness or cost benefit. However, the information gathered through these special programmes is helping in decision making in respect to treatment and control. Results obtained through survey studies and clinical approaches are used at national level for making key decisions about the socio-economic consequences of diseases caused by helminths and their control.

Table 2. List of important nematodes from veterinary perspective

S.No.	Name of the parasite	Host	Distribution	References
1.	*Ostertagia ostertagi*	Gastrointestinal nematodes (GINs) of grazing cattle	Worldwide	(Singh B. et al., 2019)
2.	*Cooperia oncophora*	Gastrointestinal nematodes (GINs) of grazing cattle	Worldwide	(Candy et al., 2018)
3.	*Teladorsagia circumcincta*	Small ruminants such as sheep	Worldwide	(Stear. et al., 2019)
4.	*Haemonchus contortus*	Attached to the abomasum of ruminants (sheep, goats and cattle)	Worldwide	(Brik. et al., 2019)
5.	*Dictyocaulus viviparous*	In the bronchial tree of horses, sheep, goats, deer, and cattle	Worldwide	(Claerebout & Geldhof 2020)

More research on prevalence, its distribution and infection mechanism is required in order to reduce the burden of parasitic diseases and to plan initiatives for its prevention and control (Redekop et al., 2017). Helminths cause significant economic losses worldwide. Due to helminths infection in cattle, overall loss has been estimated to be $ 50.67 per animal per year, which in terms of percentage, is 17.94% annually (Rashid et al., 2019). Data on economic losses due to helminths infection in humans is not available directly but the seriousness can be inferred from the fact that approximately 1.0 billion people are infected by these helminths annually (Gordon et al., 2017, Sherman, 2018).

CONCLUSION

Nematode cause considerable problems in both human as well as in domestic and veterinary animals. These infections not only cause significant deleterious effect on the life of their hosts but also cause huge monetary loss. Though impact of the diseases could be dramatically reduced by improved sanitation for humans and pasture control in domestic animals, such methods are insufficient to eradicate these parasites. In the absence of vaccines, use of chemical based compounds is the sole method to ease disease symptoms, control infection and reduce transmission. Irrespective of having WHO recommended global standard anthelmintics drugs available for treatment of nematode infections, cases of nematode infection are on the rise. The intensive and indiscriminate use of these drugs has led to widespread resistance to all current anthelmintics.

Continuous search for new anthelmintics agents and identification of new drug targets is required to overcome or prevent the issue of drug resistance. Many plant based naturally occurring compounds have been reported to possess anti-nematode potential. There is also need for well trained and skilled professionals who are capable of integrating and implementing new technologies into their countries. As the problem is global, several health agencies of various countries need to work in collaborated and coordinated manner in order to combat the disease. There is an urgent need for viable, safe and sustainable control strategies that requires an integrated approach incorporating environmental management, and includes a combination drug therapy so as to minimize the chances of parasite adaptation.

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Efficiency of Image Fusion Technique Using DCT-FP with Modified PCA for Biomedical Images

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ABSTRACT

The main issue with the multi-focus images lies in obtaining the relative information about the identification of objects in the individual images with less resolution. Hence the image fusion methods have attracted attention to obtain high resolute image with a pair of multi-focus images. The fusion methods have also raised its attention to apply for biomedical images obtained with different modalities. An attempt has been made in the present work to develop an image fusion methodology designing on discrete cosine transform using frequency partitioning (DCT-FP). For the feature extraction and for better morphological details, the paper discussed about the modified principal component analysis (M-PCA) algorithm. Six sets of medical images obtained with different modalities have been introduced to the six different image fusion algorithms including the proposed method. Various statistical metrics were evaluated for each image fusion method. The careful comparison of the visual and objective metrics reveals that the proposed method shows best performance with not only having visual quality and also confirmed based on the variation of the statistical metrics.

KEY WORDS: BIOMEDICAL IMAGES, FREQUENCY PARTITIONING, IMAGING MODALITIES, MEDICAL IMAGE FUSION, MODIFIED PCA.

INTRODUCTION

Biomedical image processing is one of the emerging tool for disease diagnosis and treatment, visualization of internal parts of the human body (Fei et al., 2017).The essence of the biomedical image processing involves in design and testing of different algorithms and helps to ease the clinical analysis at less time and also provides less space to store the processed images. Different modalities are available in medical images to provide complementary information about different parts of the body. There are certain limitations in using each modality to view the different anatomical structures of the body (Gomathi and Kalaavathi, 2016, Valdes Hernandez et al., 2010, Liu et al., 2014, Yang et al., 2014, Yin Fei et al., 2017, Rajalingam and Priya, 2018, Chen Jingyue et al., 2020, Haskins Grant Kruger Uwe and Yan Pingkun, 2020, Zhang Yu et al., 2020).

The various number of modalities are generally used in medical imaging such as MRI, CT, PET, X-ray and so on. Each modality expresses its own advantages over other modalities. For example in MRI soft tissue information is available but in CT imaging dense tissue information is provided. Like this one modality is superior in getting particular information about the human body than other (Fatma El-Zahraa et al., 2016).

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The responses from the imaging modalities provide sufficient information that can be used for the study, detection and diagnosis of diseases by radiologists. But major limitations of these modalities are that one modality cannot provide the complete information about the activity of the anatomical structures as each modality uses different radiation powers. To get benefit over all modalities, the images obtained from different modalities can be processed through image fusion algorithms to produce multimodal images. These fusion algorithms improve the identification of damaged region of the human body and also present accurate integrating complementary information useful for the clinical analysis and treatment. Rather than single modal images, multimodal images had major advantages such as combination of images of CT and MR representing both soft tissue and bone simultaneously.

Similarly, the use of image fusion algorithms in medical images MR-T1 and MR-T2 to help the segment of white matter lesions and also to guide the resection of epileptogenic lesions by neurosurgical methods. The use of PET / CT imaging provides an insight into the physiological and anatomical features of the tumor disease (Kunpeng Wang et al., 2020, Ullah Hikmat et al., 2020). Multimodality images are generally produced to get advantage over single modality image by different algorithms. One of the mostly used algorithms is image fusion algorithms. A lot of work has been carried out over the past decade in the application of appropriate image fusion algorithms for biomedical images to investigate the anatomical and physiological features of the human body.

A pulse-coupling neural network (PCNN) image fusion algorithm using sparse representation to overcome the limitations of wavelet-based transform methods and the decomposition of the coefficients of the source images was performed using NSCT method (Xia et al., 2018). Fuzzy transform (FTR) based image fusion method is proposed for multi-focus images with the help of pair of error images (Manchanda and Sharma, 2018). A cascaded PCA-based image fusion algorithm is proposed with shift invariant wavelet transform to retain important image features, such as edges, clarity, and image descriptions (Benjamin and Jayasree, 2018). An image fusion algorithm is described based on PCNN using the Poisson Hidden Markov Model (PHMM) model to implement the fusion requirements (Biswas and Kanti Sen, 2018). This hybrid algorithm improves both functional and color information of the image. NSCT and SWT based image fusion algorithm is proposed for multimodal biomedical images (Ramlal et al., 2019).

NSCT based phase congruency and local Laplacian energy is proposed for multimodal biomedical image fusion by Zhu Zhiqin et al., 2019. SPECT and CT images of the brain are considered for the image fusion using PCNN, HIS and shuffled frog leap algorithm (SFLA) (Huang Chenxi et al., 2019). A hybrid advanced image fusion method is proposed based on NSST and Parameter adaptive PCNN (PAPCNN) model (Jingming Xia et al., 2020). A two scale decomposition of images with sparse representation is used as image fusion method (Sarmad Maqsood et al., 2020). A new image fusion algorithm is proposed which takes the advantages of the transform domain such as wavelets and both Independent component analysis (ICA) and PCA methods (Satya Prakash Yadav et al., 2020). The proposed method involves the utilization of the advantages of discrete cosine transform with frequency partitioning. To evaluate the features of the fused image the paper propose a modified PCA algorithm.

MATERIAL AND METHODS

Figure 1 describes about two methods, the FPDCT-based image fusion approach (Naidu VPS,2013) and the MPCA approach which demonstrates the proposed system structure.

Image Fusion Based on DCT with Frequency Partitioning: The image fusion approach is applied with different fusion rules for high and low frequency components having partition factor ('f'). The algorithm 1 describes the different steps involved in the implementation of the proposed method.

Algorithm 1: FPDCT based Image Fusion

Input: Medical Images

Output: Fused Image

Steps :

1. Start
2. Loaded Source Images
3. To get the components of low and high frequency respectively, performed the frequency partition on source image A & source image B.
4. Using the Fusion law, images fusion is applied for low and high frequency components.
5. To obtain the fused image, the inverse DCT algorithm is applied to the high and low frequency elements of the composite.
6. Stop

The 2-D Lyapunov inequality becomes more critical option to apply for the entire frequency domain. To alleviate this it is necessary to use a matrix function varies with the frequency domain. Hence, in order to get the advantage of 2-D Lyapunov inequality, a group of discrete intervals of constant matrix functions can be considered using the frequency partitioning approach. Frequency is a periodic motion undergone for one cycle after passing through series of values. Frequency partition refers to identification of LF and HF in given set of data. Further apply frequency function 'f' which divides LF and HF values separately. Here LF indicates low frequency and HF indicates high frequency. In case of digital image, low frequency components are perceptually important. Generally, background components are considered as low frequency values.

Whereas in case of high frequency components sharp image edges are identified which represents foreground components of image. The frequency partitioning is very useful in identification of periodic texture pattern or extraction of features of image. In case of matching procedure, high frequency components are considered to identify texture pattern. With the help of 1D DCT, vector data has been generated with DCT function Z(x). Further, DCT coefficients are identified for the given vector. A partition factor 'f' is applied for energy compaction DCT coefficients (high and low frequency) which separates DCT coefficients as low frequency and high frequency separately as shown in figure 1.

$$A(p)=DCT(a(p)),p,v=0,1,2,...,XY-1 \quad (1)$$

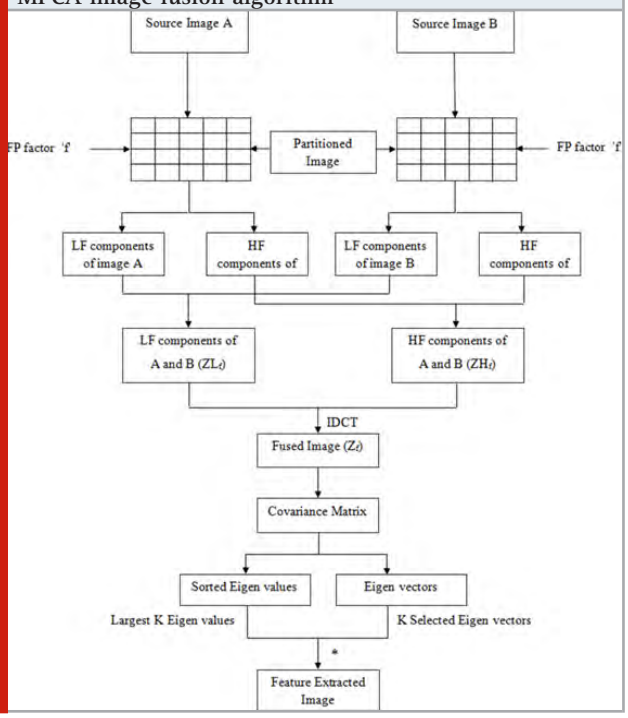
$$AL(v)=A(v),v=0,1,2,...,XYf-1 \quad (2)$$

$$A(v)=A(v),v=XYf,XYf+1,...,XY-1 \quad (3)$$

Let the images to be fused are α_1 (p,q) & α_2 (p,q) and the image fusion process is as follows:

$$\alpha_1 (p)=c2dt1d(a_1 (p,q),X,Y) \quad (4)$$

Figure 1: The flow diagram of the proposed FP-DCT based MPCA image fusion algorithm



$$\alpha_2 (p)=c2dt1d(\alpha_2 (p,q),X,Y) \quad (5)$$

$$A_1 (v)=DCT(\alpha_1 (p)) \quad (6)$$

$$A_2 (v)=DCT(2(p)) \quad (7)$$

With the help of eq. 3, the fused coefficients are represented as:

$$AL_f (v)=0.5(AL_1 (v)+AL_2 (v)),v=0,1,...,XYf-1 \quad (8)$$

$$AH_f(v)=\begin{cases}AH_1(v) \text{ if } |AH_1(v)| \geq |AH_2(v)| \\ AH_2(v) \text{ if } |AH_1(v)| < |AH_2(v)|\end{cases}, v=XYf, XYf+1, ..., XY-1 \quad (9)$$

$$A_f (v)=[AL_f (v)\ AH_f (v)] \quad (10)$$

$$a_f (p)=idct(A_f (v)), \quad p,v=0,1,2,...,XY-1 \quad (11)$$

$$\text{The fused image is: } I_f=c1dt2d(A_f (p),X,Y) \quad (12)$$

Implementation of MPCA Algorithm: The fused image obtained after applying FPDCT algorithm is given as an input to MPCA algorithm. The purpose of using MPCA algorithm is to reduce the dimensionality of the images (Savnte et al, 1987). The detailed description of some key steps involved in the MPCA algorithm is given below. The MPCA converts associated variables into a variety of unrelated principal components as a mathematical method. It determines optimum definition for compact operation of a given data set. MPCA first principle is the calculation of covariance values for a given data set. Maximum variance from the first variable of theory is determined. Let the image of the source become a vector of one column. To project the data into a 1D subspace, the following steps are required.

- Set the data to a vector.
- For the given vector, measured the matrix of covariance.
- Calculated the values of Eigen for given matrix of covariance.
- Determined the V, D is part of the Eigen function.
- Order the D as the own value decreases.
- The first column of V is computed to represent the larger value of Eigen. In order to measure P

$$P = V(: , ind(1))./sum(V(: , ind(1)) \quad (13)$$

- Eventually, to obtain image extracted as the features

$$PCA = P(1) * Img \quad (14)$$

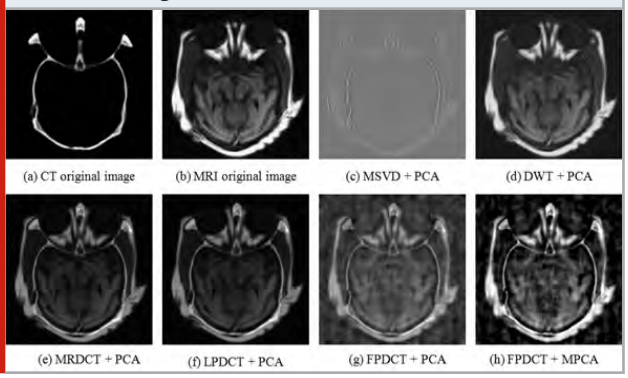
Quality Metrics of The Image Fusion Algorithm: The quality measure of the image fusion process is categorized into subjective and objective metrics. The subjective metrics usually depends on visual features and observer's professional knowledge. Further, the computation time of this process is time-consuming with poor readability. On the other hand, the objective metrics can easily be computed and calculates the similarity between the fused image and input images. The present paper evaluates twelve objective metrics to understand the performance of the proposed algorithm. (Jagalingam and Hegde, 2015, Silvina al., 2018, Yin Chen and Blum, 2009).

RESULTS AND DISCUSSION

The medical image test pairs (CT / MRI, MR - PD / MR - T1, MR - PD / MR - T2, MR - T1 / MR - T2, MR - PD /

PET, and MR - T1 / PET) were chosen by online resources such as http://www.med.harvard.edu/AANLIB/home.html page. These images were given as inputs for different standard fusion algorithms such as MSVD, DWT, MRDCT, LPDCT, FPDCT and FPDCT + MPCA (proposed method). The performance of these algorithms was analyzed using different visual and quantitative measures. The proposed algorithm fuse source images with FPDCT + MPCA process to extract features of an image.Different statistical measures such as Mean (API), QW (weighted fusion quality index), QE1 (edge-dependent fusion quality index (version 1)), QE2 (edge-dependent fusion quality index (version 2)), CQM (pistonesi_metric), and QCB (Chen-Blum metric) used to quantify the performance of the fusion algorithms mentioned.

Figure 2: Visual evaluation of the fusion of MRI and CT Medical Images using (a) CT original Image (b) MRI original image (c) MSVD+PCA (d) DWT+PCA (e) MRDCT+PCA (f) LPDCT+PCA (g) FPDCT+PCA and (h) FPDCT+MPCA.



The computed values of the statistical measures for different standard medical image test pairs using the mentioned fusion algorithms are specified in the table 1- 6. Based on the nature of these statistical measures, the values of API, QW, QE1, QE2, CQM, and QCB should be of higher value to show the enhanced performance of the fusion algorithm.

The images obtained after fusion process should be in such a way that it provide more necessary information based on people's perceptions, visual and quantitative analysis. The visual analysis of the fused image should reveal the significant improvement in the transfer of information from the source images, information lost from the source images and less artifacts. Figure 2 describes the original CT / MRI images obtained by different image fusion algorithms. The image (figure 2(h)) obtained from the proposed fusion algorithm shows better visual quality and less information loss. The statistical metrics evaluated for original CT / MRI images using different fusion algorithms are specified in the table 1. After the comparison of the statistical

Table 1. Statistical measures of medical images (CT / MRI) using MSVD+PCA, DWT+PCA, MRDCT+PCA, LPDCT+PCA, FPDCT+PCA and FPDCT+MPCA

Evaluation Index	MSVD	DWT	MRDCT	LPDCT	FPDCT	FPDCT+MPCA
API	51.7817	51.73047	32.08205	32.08356	54.22952	55.85124
QW	0.048087	0.579082	0.627186	0.627418	0.777976	0.7877
QE1	0.001948	0.280026	0.351254	0.352333	0.581518	0.5623
QE2	0.044136	0.529175	0.592667	0.593577	0.762573	0.7499
CQM	0.071625	0.614085	0.669865	0.669393	0.838948	0.8446
QCB	0.036214	0.211707	0.358095	0.34075	0.189254	0.386236

Table 2. Statistical measures of medical images (MR-PD/MR-T1) using MSVD+PCA, DWT+PCA, MRDCT+PCA, LPDCT+PCA, FPDCT+PCA and FPDCT+MPCA

Evaluation Index	MSVD	DWT	MRDCT	LPDCT	FPDCT	FPDCT+MPCA
API	37.42545	37.43239	35.36881	35.36997	42.56744	45.41017
QW	0.784108	0.837708	0.795226	0.795348	0.830738	0.8454
QE1	0.556044	0.680914	0.61139	0.612899	0.666913	0.6856
QE2	0.745683	0.825175	0.781915	0.782879	0.816647	0.828
CQM	0.829274	0.855221	0.825664	0.826336	0.853063	0.8681
QCB	0.15093	0.259611	0.208251	0.191667	0.278866	0.521908

measures obtained by different fusion algorithms, the proposed method shows good performance over other standard fusion methods expect QE1 and QE2.

The original MR - PD / MR - T1 images and images after various image fusion methods can be visualized in Figure 3. After the visual analysis of these images, the image obtained using the proposed method shows better quality and less information loss. Table 2 shows the statistical measures of the different fusion algorithms and the metrics obtained for the proposed algorithm shows better values than compared to other algorithms. Original medical MR - PD / MR - T2 images obtained after applying to different fusion algorithms are shown in figure 4. The image (figure 4(h)) obtained using the proposed method shows the better visual appearance and appreciably more image quality. Table 3 shows the quality metrics of the MR - PD / MR - T2 image using different fusion algorithms. From the visual appearance and quality metrics the proposed algorithm shows better performance than other algorithms.

Figure 3: Visual evaluation of the fusion of MR-PD and MR-T1 Medical Images using (a) MR-PD original Image (b) MR-T1 original image (c) MSVD+PCA (d) DWT+PCA (e) MRDCT+PCA (f) LPDCT+PCA (g) FPDCT+PCA and (h) FPDCT+MPCA.

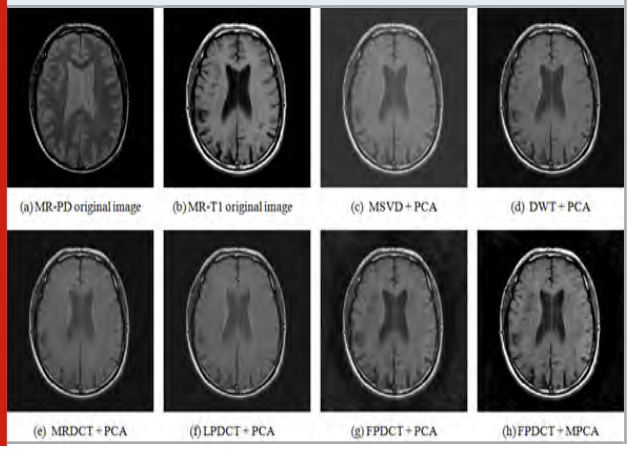


Figure 4 : Visual evaluation of the fusion of MR-PD and MR-T2 Medical Images using (a) MR-PD original Image (b) MR-T2 original image (c) MSVD+PCA (d) DWT+PCA (e) MRDCT+PCA (f) LPDCT+PCA (g) FPDCT+PCA and (h) FPDCT+MPCA.

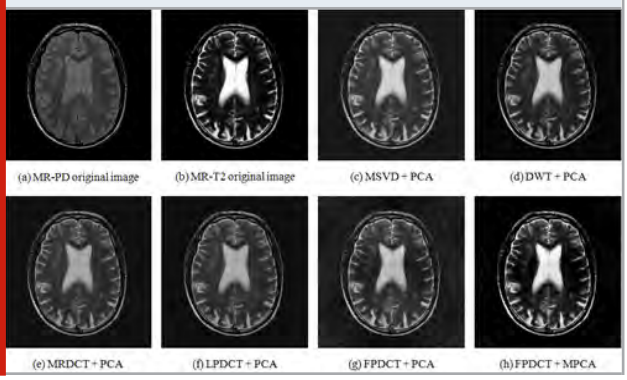


Table 3. Statistical measures of medical images (MR-PD/MR-T2) using MSVD+PCA, DWT+PCA, MRDCT+PCA, LPDCT+PCA, FPDCT+PCA and FPDCT+MPCA

Evaluation Index	MSVD	DWT	MRDCT	LPDCT	FPDCT	FPDCT+MPCA
API	28.63709	28.6367	28.53053	28.5188	28.89087	32.21809
QW	0.829519	0.903563	0.862544	0.862274	0.896868	0.9094
QE1	0.587614	0.778354	0.700519	0.700711	0.780103	0.7971
QE2	0.76656	0.882244	0.83697	0.837085	0.883234	0.8928
CQM	0.880485	0.920614	0.883243	0.883242	0.916054	0.9291
QCB	0.218126	0.303231	0.221705	0.232343	0.281452	0.553689

Table 4. Statistical measures of medical images (MR-T1/MR-T2) using MSVD+PCA, DWT+PCA, MRDCT+PCA, LPDCT+PCA, FPDCT+PCA and FPDCT+MPCA

Evaluation Index	MSVD	DWT	MRDCT	LPDCT	FPDCT	FPDCT+MPCA
API	35.53705	35.59537	35.72916	35.71688	42.56744	49.14842
QW	0.003865	0.637714	0.637597	0.639368	0.639109	0.6643
QE1	0.000003	0.419465	0.417329	0.42436	0.42573	0.4487
QE2	0.001624	0.647661	0.64601	0.651429	0.65248	0.6698
CQM	0.007306	0.667563	0.674461	0.675637	0.692676	0.7186
QCB	0.022406	0.186874	0.179976	0.155508	0.196004	0.431429

The visual information of medical MR - T1 / MR - T2 images both input and output of various image fusion algorithms are shown in figure 5. Table 4 gives the statistical measures of the fusion algorithms of the medical MR - T1 / MR - T2 image. After comparing the performance of the fusion methods, the proposed method shows good image quality and better statistical measures.

Figure 5: Visual evaluation of the fusion of MR-T1 and MR-T2 Medical Images using (a) MR-T1 original Image (b) MR-T2 original image (c) MSVD+PCA (d) DWT+PCA (e) MRDCT+PCA (f) LPDCT+PCA (g) FPDCT+PCA and (h) FPDCT+MPCA .

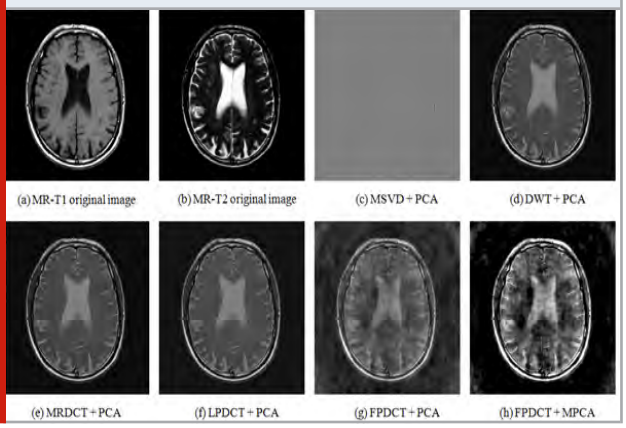


Figure 6: Visual evaluation of the fusion of MR-PD and PET Medical Images using (a) MR-PD original Image (b) PET original image (c) MSVD+PCA(d) DWT+PCA (e) MRDCT+PCA (f) LPDCT+PCA (g) FPDCT+PCA and (h) FPDCT+MPCA

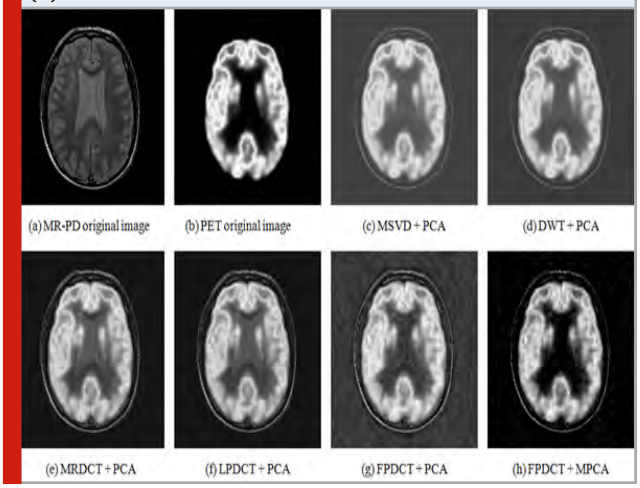


Table 5. Statistical measures of medical images (MR-PD/PET) using MSVD+PCA, DWT+PCA, MRDCT+PCA, LPDCT+PCA, FPDCT+PCA and FPDCT+MPCA

Evaluation Index	MSVD	DWT	MRDCT	LPDCT	FPDCT	FPDCT+MPCA
API	37.3841	37.34552	34.44221	34.43369	40.71423	44.66777
QW	0.581465	0.56562	0.541483	0.542056	0.536618	0.5932
QE1	0.158264	0.172488	0.171794	0.173742	0.192043	0.1984
QE2	0.397824	0.415316	0.414481	0.416824	0.438227	0.4454
CQM	0.601001	0.60154	0.570198	0.569143	0.567141	0.6257
QCB	0.157026	0.165922	0.234127	0.236401	0.237634	0.502601

Table 6. Statistical measures of medical images (MR-T1/PET) using MSVD+PCA, DWT+PCA, MRDCT+PCA, LPDCT+PCA, FPDCT+PCA and FPDCT+MPCA

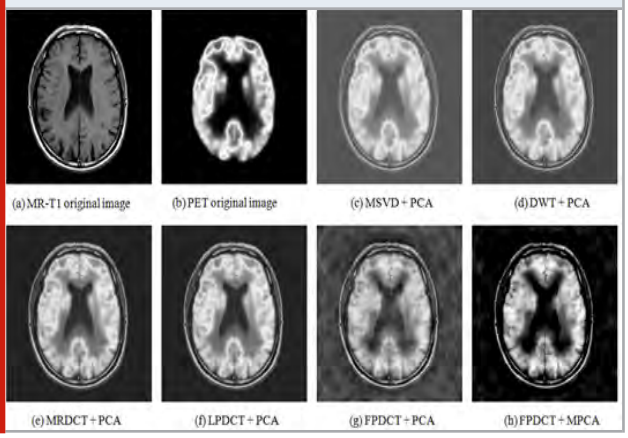
Evaluation Index	MSVD	DWT	MRDCT	LPDCT	FPDCT	FPDCT+MPCA
API	41.43909	41.45071	41.64084	41.63403	42.56744	50.32249
QW	0.343233	0.346612	0.365363	0.365374	0.289679	0.3647
QE1	0.071484	0.097302	0.106583	0.106683	0.109173	0.1209
QE2	0.267365	0.311933	0.32647	0.326624	0.330413	0.3477
CQM	0.379906	0.389641	0.399926	0.399008	0.324854	0.4051
QCB	0.131176	0.154801	0.210985	0.209323	0.222449	0.472058

The visual information of medical MR - PD / PET images both input and output of various image fusion algorithms are shown in figure 6. Table 5 gives the statistical measures of the fusion algorithms of the medical MR - PD / PET image. After comparing the performance of the fusion methods, the proposed method shows good image quality and better statistical measures.

Figure 7 shows the medical MR - T1 / PET images of various fusion algorithms. The fusion image obtained using proposed method shows better visual quality and appreciably no loss of information. Table 6 gives the information about statistical measures of the MR - T1 / PET image processed with different image fusion algorithms. The comparison of the processed fusion images and statistical measures reveals that the proposed method shows better performance than other algorithms.

After thorough investigation of the visual quality and objective evaluation metrics of the images fused by the proposed method, it is evident that the quality of the fused images is efficiently enhances and the appearance of artifacts and regions blurred in the fused images are quite significantly reduced than compared to other transform methods with and without PCA methods. The proposed method produces outperformed results are also produces outperform results than compared to algorithms proposed recently (Zhu Zhiqin et al, 2019, Yin Fei et al., 2017, Liu Xingbin Mei Wenbo and Du Huiqian, 2018, Bin Yang Chao Yang and Guoyu Huang, 2016).

Figure 7: Visual evaluation of the fusion of MR-T1 and PET Medical Images using (a) MR-T1 original Image (b) PET original image (c) MSVD+PCA (d) DWT+PCA (e) MRDCT+PCA (f) LPDCT+PCA (g) FPDCT+PCA and (h) FPDCT+MPCA



CONCLUSION

Image fusion methods have great importance to get multi-resolute image from the multi-focus images of the same scene. A new fusion algorithm based on discrete cosine transformation with frequency partitioning has been attempted. The image extraction feature was made using the modified principle component analysis algorithm, which is the heart of the proposed method. From the standard data base, various medical images with various modalities were selected and applied to different forms of image fusion methods. For the image fusion algorithms the visual and objective analysis was performed. The results show that the FP-DCT is quite efficient fusion algorithm than the other standard fusion algorithms using a modified PCA.

Conflict of Interest Statement: The author(s) declare(s) that there is no potential conflict of interest relevant to this article was reported.

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The Ability to Correct a Persons Posture with Regular Exercise

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ABSTRACT

Exercise has always been regarded as a powerful stimulant of physiological processes in the human body. It has been observed that physical exercise can have a positive influence on the posture of people of all ages. The available data are scattered and further development of research in this area was in need of synthesis and interpretation. To resolve this situation was carried out the present study, material for which was 34 sources. The methods used in the work were the bibliographic search method, the dialectical method and the synthesis method. With their help, the generalization, the comprehension and processing of the available information about the recreational opportunities of regular physical activity in relation to posture. It was found that systematic and graduated physical exercise always strengthens the back muscles, improve their tone and improve blood circulation in them. This gives grounds to consider them an affordable and effective way of correction and prevention of violations of posture. The main condition of success in this matter is the need for individual selection of physical exercises. Achieved through regular exercise correction of posture is due to the physiological load transfer on different parts of the spine, straightening it and election training the muscles of the body. Due to existing violations of the first stop progressing and then weaken, and then disappear. Achieved the positive effect of regular physical exercises is possible in the case of their conduct, providing simultaneous occurrence of positive changes in many body systems. Of particular importance here is the optimization of parameters of blood and nervous system. Apparently, the dynamics of these systems in the future can consider as the marker of success of the beginning of the application of physical loads, including posture correction.

KEY WORDS: POSTURE, SPINE, MUSCLE ACTIVITY, PHYSICAL ACTIVITY, HEALTH.

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INTRODUCTION

Posture is a comprehensive indicator of the functional state of the body (Vasilieva, 2016). Its disorders are formed under the influence of various mechanisms and often indicate the appearance of serious distress in the body. The elimination of these causes is the basis for the effective correction of posture and the prevention of its deterioration in the future (Boldov et al., 2018; Vatnikov et al.,2019).

It is recognized that such effects should be primarily aimed at the systematic strengthening of the muscles of the trunk and improving the functioning of the cardiovascular and respiratory systems (Bikbulatova, 2018a). It is also possible to achieve a beautiful posture with the help of a systematic recovery of the internal organs and spine. Improving physical culture, which significantly strengthens the muscles of the back, is considered very effective in this regard (Epifanov and Epifanov, 2008; Medvedev and Gamolina, 2008). At present, sports and fitness technologies have been seriously improved. They have become very high-tech due to the fact that they incorporate the results of many scientific studies of fitness and health (Krutsevich, 2003; Medvedev and Kumova, 2007b).

In recent years, various options for new exercises have been widely introduced into the practice of health-improving physical education, often with the use of weights that allow you to purposefully act on a separate organ and functional system. The special value of such exercises is that by applying various exercises, they can be dosed according to the strength, pace and amplitude of movements. They are able to develop muscle strength and endurance, develop joints and eliminate the effects of physical inactivity. Often, such exercises are performed on simulators, which allows you to impact on specific muscle groups and joints. The high efficiency of such exercises requires the continuation of their improvement to increase the degree of directed influence on the necessary parts of the spine, (Kashuba, 2003). Purpose of the present was to consider the main effective options for physical exercises that can affect the state of posture.

MATERIAL AND METHODS

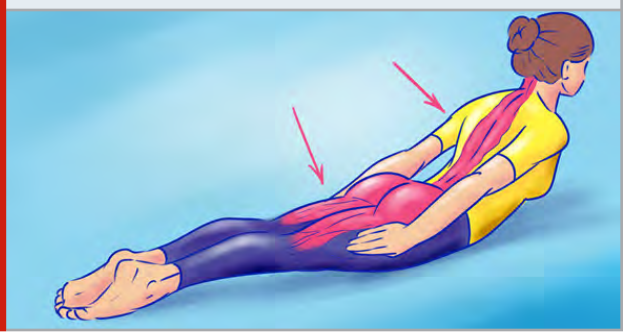
To collect data on the study topic were searched in data bases Web of Science and Scopus. Material for this study was 34 literature sources given in the bibliography. As methods the studies used a number of methods.Method bibliographic search - method of searching information sources (documents and publications), which have or may contain the desired information. The use of the method to ensure the quality of the work, as he allowed at the optimum time to obtain all the necessary information in the traditional information environment. This method was necessary for the authors to collect adequate information in modern conditions the rapid growth of the information environment of research and development.

The dialectical method is a method associated with the divergence and convergence of the whole and parts, main and secondary, essential and accidental, of statics and dynamics, abstract and concrete. The use of the dialectical method helped the authors of any phenomenon is to consider the duality of its properties and characteristics, to find their contradictions and the relationship (causality, unity, dependence). With it the properties of any phenomena splits into opposites and brought to researchers in the form of General and special quality and quantity, cause and effect, content and form. Synthesis method-theoretical-empirical method. He helped the authors to connect previously isolated parts of the object together. The connection of the results of the studies previously published works to a single system to form the most complete picture on the available scientific information.Using these methods the authors conducted a synthesis, interpretation and processing of available information, the results of which are posted in the articles section "Results and discussion".

RESULTS AND DISCUSSION

It becomes clear that systematic and dosed physical culture exercises strengthen the muscular system and therefore are the best way to prevent postural disorders. The elimination of posture disorders is a necessary condition for primary and secondary prevention of orthopedic diseases and diseases of internal organs (Karpov et al.,2018). In vivo motor activity is a combination of static and dynamic work, which is carried out against the background of tonic muscle tension, and the elements of movement create the necessary stretching and contraction (Bespalov et al., 2018b). These effects, occurring in a coordinated manner, provide a normal motor act (Medvedev and Kumova, 2007a; Stepanova et al., 2018).

Figure 1: Doing a stretching exercise to help optimize posture



Feasible muscle activity is a natural means of physiological stimulation of the body. She maintains and improves dozens of adaptive mechanisms at all levels of functioning. The work performed by the muscles is determined by the dynamics of their traction and length. Well-known types of muscle work (overcoming, inferior, holding) are determined only by the direction of change in muscle length: shortening, lengthening, maintaining length. For these three types of work (the first two are dynamic, the last is static) there is the possibility of

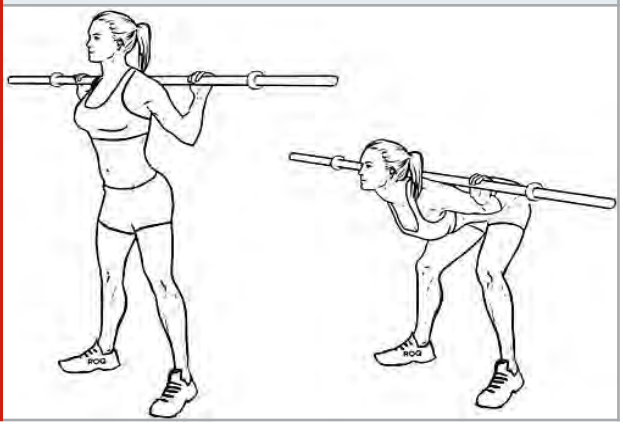
three options for changing the muscle traction force compared to the isometric one: its increase, decrease and maintenance without changes (Makhov and Medvedev, 2018c).

The use of stretching exercises contributes to the morphological restructuring and improvement of the elastic properties of pathologically altered tissues that limit the amplitude of movements or cause deformation (Figure 1) (Makhov and Medvedev, 2018a). The essence of the use of strength exercises for health purposes is to use the micropump function of skeletal muscles, which, when contracting, squeeze blood into the vessels, and when relaxing, they attract it, that is, they perform the function of the so-called "peripheral hearts" (Makhov and Medvedev, 2018b).

It is known that skeletal muscles have elastic and elastic properties, significantly contributing to the stretching and contraction of the muscle in vivo (Mal et al., 2018). However, muscle elasticity is imperfect. At the beginning of the stretching, the muscle exerts insignificant resistance to the tensile force, with further stretching, the muscle resistance of the tensile force grows. The non-linearity of muscle tension depends on the fact that in the muscle some sections are contracted, while others that are still at rest are stretched. The optimum of this process is capable of forming psychological comfort in a person (Bikbulatova, 2018b).

During exercise, the mechanical action of the muscles appears as a pull applied to the place of their attachment. The main condition determining the physiological effect of muscle traction is the load. Without a load for a muscle, there cannot be its tension and there cannot be its traction. It was noted that if a muscle is stretched repeatedly at short intervals of time, then its length increases more than with a single stretch (Makhov and Medvedev, 2018d).

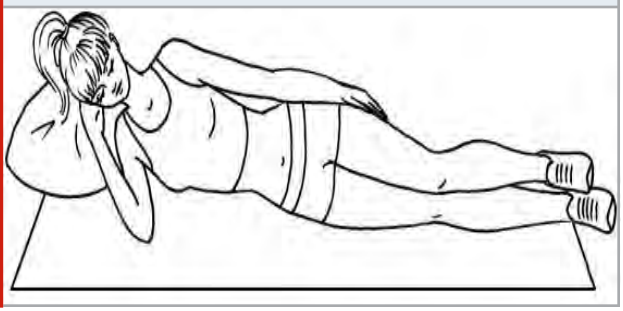
Figure 2: An example of exercises to improve posture with a gymnastic stick



Very important for improving posture is the exercise with gymnastic objects: sticks, dumbbells, balls, shock absorbers (Makhov and Medvedev, 2018e). This type of load is a physical exercise with local and dosed power stress, aimed at stretching the muscles, their relaxation, coordination of movements, as well as corrective and breathing exercises (Figure 2). The positive effect of exercises with objects on the body is more pronounced compared to similar exercises without objects due to the effect on the muscles of the mass of the object, its effect on the body as a lever due to the appearance of inertial forces during swing and pendulum-like movements (Pechenevskaya and German, 2017; Vlasova, 2017).

Special simulators used for various disorders of posture in the form of block apparatuses provide a pronounced effect due to the clear localization of the effect on the back muscles and due to the achievement of their long-term effect of tensile or tensile effects (Kazantseva, 2012). Very important for the effective correction of posture during exercise with weights is the ability to dose the load with a fairly high degree of accuracy. Such exercises make it possible to select schemes for individual training loads, taking into account the existing physical, psychological and age-related features. It is also possible to correct individual loads in the direction of decreasing or increasing, based on the initial level of training and the state of health of the student (Bespalov et al.,2018a).

Figure 3: Performing breathing exercises on the side



During posture correction, metered force loads are often applied in the form of repeated lifting of unsaturated weight to severe fatigue. The success of this method is due to the fact that the amount of work used causes significant shifts in the metabolism, creates opportunities for enhancing anabolism and leads to functional hypertrophy of the desired muscle group and an increase in their strength (Butova and Masalov, 2011).

For the formation and strengthening of correct posture, as a rule, make special corrective physical exercises which is able to adjust the arc of curvature in the spine. For this purpose, asymmetric exercises based on correction of the spine. They optimize the curvature, moderately stretch the muscles and ligaments on the concave arc of curvature and differentially strengthen the weakening muscles on the convex side. These exercises are very successful in terms of the unilateral strengthening of torso muscles.

Quite effective is also symmetric exercises that have minimal biomechanical impact on the curvature of the spine. When they are executed is not required, given the complex biomechanical working conditions and strain

of the locomotor system. This eliminates the risk of incorrect use. Symmetric exercises have different impacts on the symmetrical muscles of the trunk, resulting in spinal deformity are physiologically unbalanced state. The weak muscles of the trunk (e.g., on the convex arc of curvature) at each symmetric activity should have increased functional capacity, consequently they train harder than stronger muscles (Karpov et al., 2020).

It is not uncommon for posture correction applied detorsion exercises aimed at correcting the existing deformities and prevention of disorders of the spine. In these exercises, the unloading of the spine appears as a necessary moment of the General and local effect. The most common unloading position is horizontal. Lying down relieves tension of the muscles and spine can give physiological position changes the localization of the center of gravity, and the body acquires most of the footprint, providing stable equilibrium (Zemba and Morozova, 2009).Symmetric and asymmetric exercises are mainly used to influence the spine in the frontal plane. To influence the deformation in the horizontal plane, special corrective exercises of a derotational nature are used. However, this type of exercise has not found wide application, since the peculiarities of the muscle reaction to the load, carrying out the movement of the spine in a horizontal plane, have not been studied enough (Osipov and Bulanova, 2008).

In case of posture disorders, breathing exercises have been very actively used recently (Figure 3). They normalize the breathing process and coordinate breathing and movements, strengthen respiratory muscles, improve chest and diaphragm mobility, and prevent and correct chest deformation. Particular attention should be paid to deep breathing exercises that provide physiological conditions for the work of the respiratory muscles and have a restorative effect. It is very advisable to introduce breathing exercises in the initial position while lying on your side. They increase intercostal spaces. This position of the body has a moderate detorsion effect on the vertebrae and ribs from the concave side (Tsykunov, 2018).

The optimal exercise program for posture correction should be designed in such a way as to train the body and prevent the possibility of chronic exhaustion in it. In the process of doing the exercises, the resulting fatigue should increase gradually, while the moment of termination of the load in each case should be determined individually. One should strive to ensure that the next load is performed after achieving full recovery from previous physical work, not allowing the beginning of the next load, until the moment of full recovery after the previous work, since it is very dangerous for the development of chronic exhaustion (Gimazov and Bulatova, 2013).

A very important health-improving mechanism of regular physical exercises in case of posture disorders and scoliosis is their positive effect on hematological parameters, especially those related to blood microreology (Medvedev et al.,2010). It has been established that the optimization of the state of muscles in the body, including in the paravertebral zone, occurs due to the positive dynamics of the properties of red blood cells and platelets, the movement of which through the vessels is very significantly responsible for metabolic processes in them. It is noted that regular muscle activity reduces their aggregation and improves the surface properties of the membrane (Medvedev, 2018a). There is some evidence that the activity of red blood cell aggregation is associated with the intensity of the tested physical activity and their nature. In people who regularly experience significant physical activity, the aggregation of red blood cells and impaired surface properties of their membranes can often be lower compared to those who do not engage in sports (Mal et al., 2019).

In addition, regular muscle activity lowers platelet activity to a lower physiological level (Medvedev, 2018b). In a significant number of cases, these effects were traced on productive animals in loose housing. At the same time, the similarity of the functioning mechanisms of platelet hemostasis in all mammals makes it possible to take into account the results of these studies when understanding the effects of physical activity in humans. Low platelet aggregation in vitro and in vivo on the background of dosed physical activity indicates their positive effect on platelet hemostasis (Zavalishina, 2018a). The achieved effects are due to improved metabolic processes and optimization of lipid peroxidation in plasma and platelets (Zavalishina, 2018b). This is due to the development of platelet receptor rearrangements and physiologically beneficial changes in plasma protein composition.

CONCLUSION

Exercise is a powerful biological stimulant for most physiological functions of the body. This allows you to use dosed physical activity in order to increase the volume of any muscle groups. Feasible physical exercises also cause simultaneously positive changes in the nervous, endocrine, cardiovascular, respiratory and excretory systems. With the help of special physical exercises, you can successfully regulate the functional state of the body. Very often, such exercises are used for violations of posture. When they are performed, the vertical position of the spine is optimized, the hematological parameters of the body that provide trophic muscle, including paravertebral, are improved. The redistribution of loads on the structures of the spine with the help of exercises is able to provide correction of posture due to selective training of the muscles of the trunk, increased hemocirculation in them and normalization of the condition of the spine. Regular physical exercises for violations of posture should always be aimed at preventing its progression and correcting existing defects. Very successfully, this effect is achieved when performing physical exercises with weights.

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Role of Informed Consent in Psycho-Social Research and Clinical Practice

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ABSTRACT

Informed consent' (IC) is a mandatory document prioritized before execution of any invasive procedures, life threatening test, risky methods and protocols, intimate examinations and minimal to major surgeries. Application of IC is called 'Informed Consent Process'. IC plays vital role in both, research and clinical practice of healthcare domains for protection of patient's legal rights to guide ethical approach and the procedures to be carried out. IC enables patient to choose the choice of treatment/procedure in clinical practice and subjects/participants in research (if conscious). In case of unconscious patient's, family members/relatives/guardians rule the mode of action for procedures. Looking back in history, emergence of IC dates back to over a century ago started with the aim to protect subject/patient from unwarranted intrusions into their body thus providing discretion in selecting treatment as a personal choice. As research amplified with passage of time, IC became a major part in all research activities and also clinical trials or practice simultaneously to avoid misuse of an individual. Before implementation of an IC, certain pre-requisite are to be present without failure following which if not available, the concerned act shall either be not be allowed to be carried or postponed till the necessary consent is achieved. These are strictly followed across the globe and stated as individual being an adult (more than 18 years of age), conscious, alert, mentally sound, responsive to verbal and visual commands. In procedures or treatments involving children as subjects or patients, parents/guardians/relatives should pose the same qualities. While in case of capacitated individuals (children or adult) family members/guardians/relatives are expected to be present on the spot before instigating the components of the IC. In the present article, author would concentrate on all components to be considered while de-signing an IC. Modifications according to perception either for research or clinical practice should be implemented accordingly.

KEY WORDS: INFORMED, CONSENT, PROCESS, RESEARCH, CLINICIAN, COMPONENT, TREATMENT.

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INTRODUCTION

'Informed consent' (IC) is a mandatory document prioritized before execution of any invasive procedures (Rao, 2008), life threatening test, risky methods and protocols, intimate examinations (Habiba et al., 2004;Sepucha et al., 2007) and minimal to major surgeries (Sharp., 2004; Braddock et al., 2008; Black et al., 2009) in case of emergencies (Akkad et al., 2004; Akkad et al., 2006) or routine procedure. Application of IC is called 'Informed Consent Process'. IC plays vital role in both, research and clinical practice of healthcare domains for protection of patient's legal rights to guide ethical approach and the procedures to be carried out (Hall et al., 2012). IC enables patient to choose the choice of treatment/procedure in clinical practice and subjects/participants in research (if conscious) (Bhatt, 2015). In case of unconscious patient's family members/relatives/guardians rule the mode of action for procedures. Looking back in history, emergence of IC dates back to over a century ago started with the aim to protect subject/patient from unwarranted intrusions into their body (Sutrop, 2011) thus providing discretion in selecting treatment as a personal choice (Dankar et al., 2019).

As research amplified with passage of time, IC became a major part in all research activities (Dankar et al., 2019) and also clinical trials (Kass et al., 2015) or practice simultaneously (Dankar et al., 2019) to avoid misuse of an individual. Before implementation of an IC, certain pre-requisite are to be present without failure following which if not available, the concerned act shall either be not be allowed to be carried or postponed till the necessary consent is achieved. These are strictly followed across the globe and stated as individual being an adult (more than 18 years of age), conscious, alert, mentally sound, responsive to verbal and visual commands. In procedures or treatments involving children as subjects or patients (Miller, 2009), parents/guardians/relatives should pose the same qualities. While in case of capacitated individuals (children or adult) family members/guardians/relatives are expected to be present on the spot before instigating the components of the IC. In the present article, author would concentrate on components to be considered while designing an IC. Modifications according to perception either for research or clinical practice should be implemented accordingly.

While designing and preparing an IC, various parameters dominating the fields of research and or clinical practice have to been taken care of and explained to the subject/patient (Kadam, 2017). The components to be expressed without failure shall be discussed in points for the ease of future readers, researchers and clinicians.

1. Clear terminology expressing the pathology/dysfunction/disorder/disease/condition/deformity, the patient is sufering from should be the foremost component of a professionally sound IC (Rao, 2008; Sreenivasan, 2003; Pandiya, 2010)

2. Necessity for the procedure in consideration along with the emergence required to perform the same should be attentively addressed (Rao, 2008).

3. The normal route of containment and spread of condition (if any) should be explained (Michie and Lester, 2005).

4. All temporary and permanent complications, risk, discomforts etc. associated with the procedure/treatment in context to the prevalent condition should be narrated in simple and patient's understandable language. In addition, any effects which can be presumed to originate in future should also be discussed enabling the concern to concentrate his temperament in tolerance with the complications (Madhava, 2000; Kharawala and Dalal, 2011; Gupta and Kharawala, 2012).

5. Side effects following non participation in the recommended procedure and their impact on the individual's psychological, social, mental and financial wellbeing should be prioritized (Rao, 2008).

6. Detailed information regarding all treatment strategies available within reach of the patient should be explained (De Costa et al., 2004) In addition, procedure far from patient's reach should at least be put to discussion, as sometime patients seeking long term effects of the procedure enhancing their quality of life take financial support from sources and go ahead with the higher benefited procedures.

7. Benefits and risk associated with the procedure/intervention maintaining the hierarchy with the treatment/procedures with minimum risk should be explained first following moderate to the ones ofering the least. This procedure can be used vice-versa according to the researcher/clinician choice depending on their convincing ability to make the participant/patient ready for the considered activity (Hudak et al., 2008).

8. Duration of treatment (Madhava, 2000) covering both, in-patient at the hospital/nursing home/clinic etc and out-patient department for the procedure should be explained.

9. Confirmative cost of treatment should be openly and clearly discussed with the patient (if conscious), while if the patient is unconscious, family members/relatives/friends should be explained the same, ensuring their decision to be the final for betterment of concerned individual.

10. Expected outcomes from the procedures/treatments should be explained via verbal communication. If required use of pictures, graphics and flow chart representation for a clear image of the expression should be instituted by the health staff intends to convey the information written in the IC.

11. Cost and days required for follow up wherein post treatment, the number of days to be required as in-patient

and later continuing as out-patient department should be narrated effciently.

12. Statement stating benefits that could be incurred by the individual during and on completion of procedure/ treatment should be wisely addressed.

13. Information regarding storing of records/data (Francis, 2004; Surendra and Mohan., 2017; Ohmann et al., 2017) by either the individual or health staff should be addressed with the final approval mentioned in the IC to prevent future conflicts between the individual and health care professional concerning disruption in privacy.

14. A provision of 'anonymity' (Beauchamp et al., 2001) should be created in an IC to increase the rate of participation for conducting research procedures and clinical trials. This aspect relates to acts involving patient's emotional, physical and personal attributes being a major concern for affecting the rate of participation in studies. People in developed countries being open minded in attitude respond more and faster to procedures with minimal or no anonymity, but population from developing countries (Fitzgerald et al., 2002) are usually seen to be hesitant and recommend maintenance of anonymity if asked to be a part of the procedure/treatment.

15. Disclosure of small to large remuneration during the entire course should be addressed as focusing this concern in later stages of IC, as initially the major concern should be to convince individuals buy showing the benefits of the procedures/treatment on their health which is major part of ethics in healthcare. Still if individuals are not willing to be part of the act, if approved by the ethical committee of the university/college/hospital/agency, remuneration can be ofered, but this is not mandatory to be part in all IC forms. Remuneration providing financial support lures individuals to participate without putting any burden on self-finances.

16. Details of contact personal in case of emergency should be documented making sure that the personals are adult in nature (more than 18 years). The concerned should provide their telephone numbers and address. A minimum of 2 references should be maintained in the IC.

17. A statement stating consent for clicking photographs, if need to be clicked during and after the procedure should be clearly mentioned. In addition, the use of the photographs to be for either educational/symposium/ conferences by the researcher or clinician should be mentioned. Provision of blindfolding of face should be explained. If not addressed in the IC, later it can put the re-searcher/clinician in legal trouble if the individual proves the expression of photographs in public without his/her consent.

18. Explanation of all technical, specific and complex words should be discussed and explained in the individuals easy and layman understandable language (Pandiya, (2010); Bhansali et al., 2009). If required, the same details can be explained to the attendants who can try to convince for the procedures to be implemented for betterment. If still the individual finds being in dilemma, videography (Wirshing et al., 2005); Deyo et al., 2000; Jimison et al., 1998) to explain the aspects can be used for a thorough and clear representation.

19. Apart from the above components necessary to be documented in an IC, any consideration in alteration to psychological (Kharawala and Dalal., 2011); (Gupta and Kharawala., 2012), physical, emotional (Kharawala and Dalal., 2011); (Gupta and Kharawala., 2012) and financial distress should be discussed without failure to prevent keeping the individual in darkness for self benefit by the researcher/clinician (Nijhawan et al., 2013).

20. Finally, a statement regarding final acceptance keeping in view of all explained factors and parameters associated with the present and future, procedures/test in case of research and treatment in case of pathology/ condition/dysfunction/ailment should be stated in the last under which the participant is asked to provide a signature or a thumb impression if illiterate.

CONCLUSION

An IC for research/clinical practice should address and document concerns in clarity stating the benefits to the individual involved during and after completion of the procedure or treatment. It should be matured enough to contain and preserve the ethical spirit by preventing misleading of the individual thus, averting any possible legal conflicts in future. This goal can only be achieved when a well contented IC form with all necessary information and in depth inoculation is designed and eventually brought in use. This well designed IC protects both, the individual and health care professional from legal; aspects giving full description of the patient to choose the type, time of treatment, refusal and withdraw from the procedure at any instinct of time during both, invasive and non-invasive surgeries. Finally, a clear presentation of a procedure is important for an individual who lets an external person to intervening in their body. This procedure should be executed using tremendous care and safety measures for betterment of the individual.

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A Comparative Study of Urinary Proteins Using Different Precipitation Methods

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ABSTRACT

Urinary proteomics is the large scale characterization of the protein content of the urine of any organism which is widely used for the diagnosis of a numerous number of human diseases. The ultimate goal of urinary proteome analysis is to compare the protein profile of the normal individual with diseased, so that the indifferent expression of the protein can be identified, finally leading to the designing and development of an innovative biomarker with a diagnostic value. The present study is aimed at standardizing a protocol for the precipitation of the total urinary protein, which can yield high protein concentration. Different precipitation methods of protein precipitation using the TCA, Acetone and Acetonitrile have been employed for extracting the total protein content of the urine. The proteins extracted from the various procedures were validated quantitatively and qualitatively using colorimetric analysis and SDS PAGE respectively. The results of the validating processes have proved that the precipitation of the urine with Acetone precipitation resulted in better recovery and integrity of proteins.

KEY WORDS: BIOMARKERS, PROTEIN PRECIPITATION, URINARY PROTEINS.

INTRODUCTION

A proteome is a complete set of proteins that is normally expressed by the organism, at a specific time in a specific cell or tissue. The proteome can change with time with respect to changes in the physiological behavior of cells, (Anderson et al., 2016, Ghosh et al., 2016). Proteomics is used to characterize the entire protein complement of an individual which deals with the study of expression, interactions and functions of proteins(Aslam et al., 2017). The genomics research has provided information on the protein encoding genes that are expressed by the numerous cell types of the human body. The human proteome research is the next step to be taken forward from the information provided by the genomics research,(Uhlen, 2008). The applications of proteome research is applied in understanding the protein expression, protein interactions and post translational modifications, (Aslam et al., 2017, Chen, 2017, Bonislawski, 2020).

A biomarker is a biological component characteristic to any individual which can be measured and appraised as a useful tool to indicate the normal biological processes, disease conditions, or effect of the drug response during

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therapeutic process(Strimbu and Tavel, 2010). A good biomarker should be accurate, non-invasive and easily collectable for performing the tests(Selleck et al., 2017). Urine is a potential source of a wide variety of components ranging from the small molecular weight metabolites and peptides to high molecular weight macromolecular complexes like exosomes, small vesicles and cytosolic proteins (Hildonen et al., 2016). In view of this, urine can be used as an excellent tool for the analysis of biomarkers because of its simple composition, abundant availability and frequent sampling from the same individual, (Lin et al., 2018).

Presently, existing tests can be used to determine either the level of total protein or a single protein in urine, whereas the evolving technology of proteomics facilitate the instantaneous analysis of the multiple urinary protein patterns and their link with the process of diagnosis or the individual's response to any treatment(Amiri-Dashatan et al., 2018). There are various processes available by which the proteins of urine can effectively be isolated and used for the diagnosis of the diseases. Fractionation of the proteins can be done by employing techniques like chromatography, electrophoresis, gel filtration column, dialysis, centrifugal separation or precipitation,(Potts, 1965). Precipitation methods are the most commonly employed method for separation of proteins from the biological samples. The present study involves the evaluation of various methods of precipitation which can be used for efficient precipitation of the urinary proteins in large quantities.

MATERIAL AND METHODS

Sample Collection: About 50 – 100ml of the clean, first urine sample in the morning from 4 male and 4 female healthy volunteers (between 20 to 40years of age) was collected in a sterile polypropylene centrifuge tubes as per the method described earlier (Thongboonkerd et al., 2002).

Precipitation of total proteins from urine: The total proteins of the urine samples were fractionated by different precipitation methods. Total nine methods have been employed, three based on TCA precipitation (Magistroni et al., 2009), four based on Acetone precipitation (Magistroni et al., 2009) and two based on Acetonitrile precipitation (Polson et al., 2003). All the methods are different from each other on the basis of i) temperature and duration of incubation, ii) duration and speed of centrifugation, iii) number and duration of washing step, iv) type and volume of solvents used.

TCA precipitation

Method 1: 50ml aliquot of urine sample was mixed with 12.5ml of 85% ice-cold TCA and incubated at 4°C overnight in a refrigerator. The mixture was then centrifuged at 8000xg for 20min at 4°C and the pellet was washed with 85% ice-cold acetone. The re-suspended pellet was again centrifuged at 12,000xg for 15min at 4°C and the resultant pellet was air dried, re-suspended in 2ml of PBS (Phosphate buffer saline) & 50µl of 8% phenylmethylsulfonyl fluoride (PMSF) (protease inhibitor) followed by storage at -20°C for further analysis.

Method 2: An aliquot of 50ml urine sample, mixed with 6.5ml of 85% ice-cold TCA followed by incubation on ice for 2h and spun at 8,000xg for 20min at 4°C. The pellet was washed with 90% acetone by vortexing and then centrifuged at 12,000xg for 20min at 4°C. The pellet was collected, air dried and re-suspended in 2ml PBS & 50µl of 8% PMSF and stored at -20°C.

Method 3: To 50ml of the urine aliquot, 25ml of 85% ice-cold TCA was added, mixed and incubated at 4°C for 10min. The mixture was then centrifuged at 12,000xg for 10min at 4°C and the pellet was re-suspended in 12.5ml of 90% acetone. The resultant mixture was spun at 14,000xg for 30min at 4°C and the above step was repeated for three times. The subsequent pellet was air dried, re-suspended in 2ml PBS & 50µl of 8% PMSF and stored at -20°C.

Acetone Precipitation

Method 1: An aliquot of 50ml urine sample was mixed with half the volume of 85% ice-cold acetone, mixed well and kept on ice for 2 h. The incubated mixture was centrifuged at 12,000xg for 15min at 4°C and then collected pellet was washed with ice-cold acetone. Again the suspension was centrifuged at 12,000xg for 15min at 4°C and the pellet was collected, air dried and re-suspended in 2ml PBS & 50µl of 8% PMSF for storage at -20°C.

Method 2: 50ml aliquot of the urine sample was mixed with thrice the volume of 85% ice-cold acetone and kept at -20°C for 30 min. After incubation, the mixture was centrifuged at 13,000xg for 20min at 4°C and the pellet obtained was washed with ice-cold acetone for 3times followed by centrifugation. The pellet thus obtained was air dried and re-suspended with 2ml PBS & 50µl of 8% PMSF before storage at -20°C.

Method 3: To 50ml of the urine sample, twice the volume of 85% ice-cold acetone was added, mixed well and incubated on ice for 2h. The mixture was then spun at 12,000 x g for 30min at 4°C and the pellet collected was air dried. The dried pellet was re-suspended in 2ml PBS & 50µl of 8% PMSF before the storage at -20°C.

Method 4: 50ml of the urine sample was taken in a centrifuge tube and equal volume of 85% ice-cold acetone was added, followed by incubation overnight at -20°C. The mixture was centrifuged at 12,000xg for 20min at 4°C. The supernatant was discarded and the pellet obtained was air dried, re-suspended in 2ml PBS & 50µl of 8% PMSF. Sample was stored at -20°C until the further use.

Acetonitrile precipitation

Method 1: Four times the volume of ice-cold acetonitrile was added to a 50ml aliquot of urine, followed by incubation at room temperature for 90min. After incubation, the mixture was centrifuged at 12,000xg

for 15min at 4°C and the pellet obtained was allowed to air dry. The pellet was then re-suspended in 2ml PBS & 50µl of 8% PMSF and stored at -20°C.

Method 2: To 50ml aliquot of the urine sample, equal volume of ice-cold acetonitrile was added and then left on ice undisturbed for 2h. The incubated mixture was then spun at 10,000xg for 15min at 4°C and the pellet collected was air dried. The final pellet was re-suspended in 2ml PBS & 50µl of 8% PMSF followed by storage at -20°C.

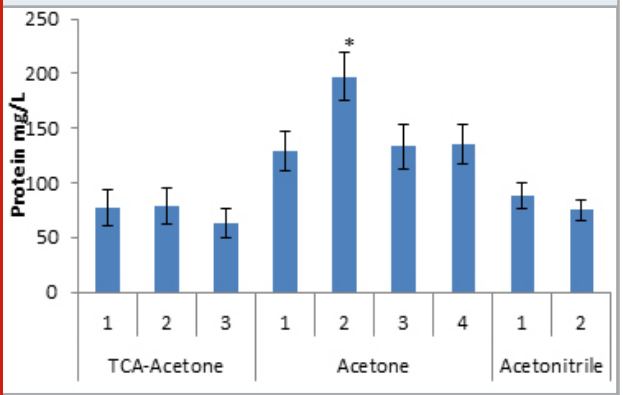
Estimation of total protein content: The total protein content of the fractionated urine samples were estimated by the method of Lowry (Lowry et al., 1951). Protein was estimated using standard curve prepared by BSA (Bovine Serum Albumin) in the concentration of 200µg/ml.

Qualitative analysis of urinary proteins using SDS PAGE: The urinary proteins extracted by the precipitation methods were analyzed qualitatively by SDS-PAGE (He, 2011). The protein fractions were subjected to separation through 10% Acrylamide gel. The separated bands were then stained using Coomassie Brilliant Blue (CBB) staining.

RESULTS AND DISCUSSION

The protein fractions from eight different urine samples precipitated by the nine different methods, when subjected to quantitative analysis by Lowry method, showed highest concentrations (197.3±41.6mg of protein/lit of urine) in the fraction obtained by the method no. 2 of the Acetone precipitation. The other methods of the Acetone precipitation have yielded significantly high amounts of the protein fractions (129.3mg, 133.4mg and 135.3mg of protein/lit of urine) for method 1, 3 and 4 respectively, compared to other studied methods. The TCA precipitation and Acetonitrile precipitation methods yielded low concentration of the proteins, as shown in fig. 1.

Figure 1: Concentration of urinary proteins extracted by the various precipitation methods.

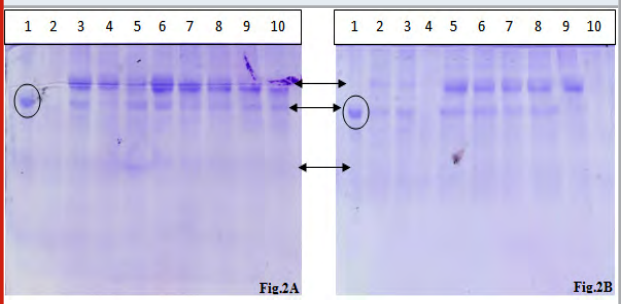


*compared to all the studied groups, p<0.005

The protein fractions of the urinary samples of the eight volunteers were analyzed on the SDS-PAGE and visualized after CBB staining. Almost all the protein fractions displayed the presence of two high molecular weight proteins and one low molecular weight protein in protein fractions.As shown in fig 2A two high quality bands corresponding to high molecular mass bands were observed with the CBB staining of the gel, in the almost all the wells except TCA-acetone method no. 1. The acetonitrile method 1 yielded two low intensity high molecular mass bands. TCA-acetone method no. 1, acetone methods 1 & 4 and acetonitrile method 2 presented one high intensity band corresponding to a high molecular mass protein.

In fig. 2B, except the TCA-acetone method no. 3 and acetonitrile method no. 2, all the other methods possessed two high intensity high molecular mass spots, out of the two bands, one was completely disappeared in acetonitrile method no. 1. High level of smearing was also observed in all the methods in high intensity, except in acetonitrile method no. 2, where the smearing is of low intensity. Two high intensity spots equivalent to high molecular mass proteins were observed in all the methods; however, one protein band was completely disappeared in all TCA-acetone precipitation methods.

Figure 2: SDS PAGE analysis of urinary proteins extracted from two different samples.



Lane 1: BSA standard,,Lane 2: TCA- Acetone Precipitation Method 1,,Lane 3: TCA- Acetone Precipitation Method 2, Lane 4: TCA- Acetone Precipitation Method 3, Lane 5: Acetone Precipitation Method 1, Lane 6: Acetone Precipitation Method 2, Lane 7: Acetone Precipitation Method 3, Lane 8: Acetone Precipitation Method 4, Lane 9: Acetonitrile Precipitation Method 1, Lane 10: Acetonitrile Precipitation Method 2

The present study takes its root from the interest in clinical proteomics and the subsequent discovery of clinically important biological markers. Thus urinary proteomics is receiving an increasing application in clinical diagnosis and follow up measure to be adopted. Even though urine proteomics gains its importance, there are some specific parameters viz., salt removal from sample, presence of minimal quantity of clinically valuable proteins, timing of sample collection, sample storage conditions and adaptation of an efficient protein sample preparation method, which plays a prominent role (Thongboonkerd et al., 2002, González-Buitrago et

al., 2007, Candiano et al., 2010, Rastegari et al., 2011). The current study employed nine protein precipitation methods in order to overcome the choice of variation in solubility, hydrophobicity, size, pI and charge of the precipitated proteins from urine (Thongboonkerd et al., 2002).

The first phase of any clinical proteomics is to find out the most efficient method for optimized purification / separation of the biomarkers from sample. In this way, for identifying the most effective method of precipitation of the proteins from urine, nine different precipitation methods using TCA-acetone (3 methods), acetone (4 methods) and acetonitrile (2 methods) were selected. All these methods differ from either of them in one or other in the following parameters: i) temperature & duration of incubation, ii) duration & speed of centrifugation, iii) number & duration of washing step and iv) choice & volume of solvents used. The total protein fractionated was quantified using Lowrys method (1951) and analyzed on SDS-PAGE. Quantitative analysis by Lowrys method (1951) showed almost similar yield in all the three methods of TCA-acetone precipitation, in case of acetone precipitation, method no. 2 resulted in higher yield of the protein, compared to all other precipitation methods. The other three methods of acetone precipitation have showed significantly high quantity of the proteins. The two methods of acetonitrile have yielded lower quantity compared to acetone methods.

However, the highest quantity of the protein yield was found to be in method no. 2 of the acetone precipitation method resulting in good recovery of the urinary proteins. The qualitative analysis of the proteins from the TCA-acetone method by SDS-PAGE revealed that the bands were either absent or, if present, they are of not intact or of low intensity. The banding patterns of the proteins were varying, in which the bands were sometimes completely absent (Contreras et al., 2008, Walliwalagedara et al., 2010). Besides the presence of some intact bands, the smearing of the bands was observed in almost all the methods. The method 1 of acetonitrile precipitation resulted in better results in terms of band visibility, intensity of the bands and resolution of the bands into intact ones in comparison to TCA methods. However, the band intensity and smearing was similar as in other methods of TCA-acetone precipitation (Walliwalagedara et al., 2010, Contreras et al., 2008).

The protein obtained from acetone precipitation method no. 2 resulted in better bands in terms of number & intensity of the bands, resolution and the extent of smearing. The TCA-acetone precipitation resulted in lower yield and poor resolution of the bands, which may be due to the presence of salt that may interfere with quantitative estimation and electrophoretic separation, and the prolonged exposure to low pH may also result in denaturation (Walliwalagedara et al., 2010), or the proteins which pI value not in the acidic range, because of the addition of TCA, may be lost during the subsequent washing steps (Jiang et al., 2004, Simpson and Beynon, 2010), or may be due to poor solubility of the proteins (Carpentier et al., 2005, Contreras et al., 2008, Walliwalagedara et al., 2010, Rastegari et al., 2011). Sometimes, extended incubation time also resulted in noticeable positive effect on the protein precipitation. However, change or increase in TCA quantity and increase in number of washing steps doesn't help much in high protein recovery.

While comparing the three precipitation methods, precipitation using acetonitrile resulted in relatively good protein recovery and good separation on the gel, which is in accordance with the results of published earlier (Romitelli et al., 2007). The acetonitrile precipitation methods also resulted in comparably good results, with variation in the number, intensity and smearing of the bands. This banding pattern of acetonitrile may be due to the property of it to remove some high molecular weight proteins (Alpert, 1999). Evaporation of acetonitrile (Sakuma et al., 1987) may influence the yield of obtained results by both the methods. Though, increase in the solvent volume does makes a sense in higher yield and retrieval of greater number of protein bands, which may have lost in the process with lower volume (Alpert, 1999, Romitelli et al., 2007).

Of all the methods, precipitation by acetone generated better results in terms of protein yield, resolution of the bands, number and intensity of the bands, amount and intensity of smearing, which is in support of earlier results too (Jiang et al., 2004, Walliwalagedara et al., 2010). This is due to the property of acetone in removing the impurities like salts, lipids and pigments that are considered as the possible parameters of interference in urine analysis (Rastegari et al., 2011). In addition, the pH of urine is acidic and that of acetone is 6.0 and this minimizes the rate of protein denaturation when compared to TCA precipitation (Thongboonkerd et al., 2002). From the analysis made during the study, the acetone precipitation method no. 2 and acetonitrile precipitation method no. 1 yielded better urinary proteins in terms of quality and better resolution of the protein bands on the gel.

In conclusion, present study explains the comparative study of precipitation methods for precipitation of urinary proteins, insight to open the door of biomarker discovery through non invasive sampling method, such as urine.

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A Novel Blood Vessel Extraction Using Multiscale Matched Filters with Local Features and Adaptive Thresholding

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ABSTRACT

Retinal blood vessel structure is an important feature for computer-aided diagnosis and treatment of diseases including diabetic retinopathy, hypertension, glaucoma, obesity, arteriosclerosis and retinal artery occlusion, and an accurate extraction is required to improve the accuracy of the diagnostic task. This paper proposes a new algorithm for blood vessel segmentation and extraction in retinal images. A multiscale matched filter combined with local features is developed to effectively extract blood vessels from retinal images. Local features are extracted from a circular and adaptive window around a candidate blood vessel pixel. Experimental evaluation using publicly available DRIVE and STARE databases shows accurate extraction of vessel networks as demonstrated by improved false alarm rates and segmentation accuracy when compared against existing works. The mean true positive rate (TPR) values obtained are (0.7661%) and (0.6312 %) for STARE and DRIVE datasets respectively, while the mean false positive rate (FPR) values achieved are (0.0311 %) for STARE and (0.0183 %) for DRIVE. Moreover, our proposed method gave high accuracy values when compared to similar work on same datasets, 93.53% and 94.73% for STARE and DRIVE datasets respectively. While in the cases of methods achieving higher accuracy value than ours, we either have a higher TPR or a lower FPR. These promising results can be enhanced in the future by deploying some other features and/or experimenting different thresholding techniques. In addition to the detection of blood vessels from retinal images, there is ongoing work to develop a quantitative method based on the shape and regularity of the blood vessels detected in order to detect possible signs / symptoms of a disease.

KEY WORDS: MULTISCALE MATCHED FILTERS, RETINAL IMAGERY, BLOOD VESSEL EXTRACTION, ADAPTIVE THRESHOLDING.

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INTRODUCTION

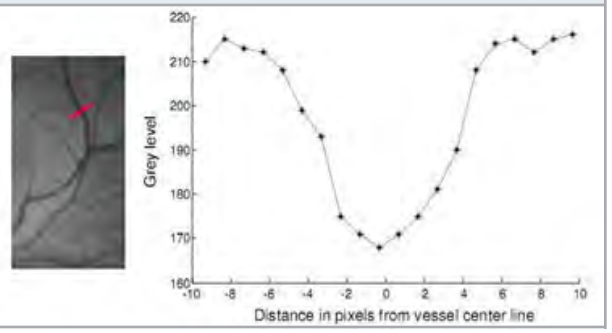
An accurate extraction of retinal vascular tree is an important task in computer aided diagnosis of retinopathy. The literature shows that there are several methods for retinal vascular tree extraction (vessel extraction) with different approaches. Based on several reviews and surveys Kirbas & Quek (2004, 2003); Mabrouk, Solouma, & Kadah (2006); Mansuri (2011), blood vessel segmentation algorithms can be divided into six main categories, which are : pattern recognition techniques, model-based approaches, tracking-based approaches, artificial intelligence-based approaches, and miscellaneous tube-like object detection approaches. Multiple techniques from the above categories are also combined and used together to solve different segmentation problems.Retinal vessels are affected by some diseases such as: diabetic retinopathy, hypertension, glaucoma, obesity, arteriosclerosis and retinal artery occlusion. Such diseases are diagnosed by studying and observing the retinal vascular tree Gao et al. (2001, Kocayigit et al 2020).

Pattern recognition techniques for retinal blood vessel detection can be further di- vided into the following categories Kirbas & Quek (2003): (i) multiscale approaches, (ii) skeleton-based approaches, (iii) region growing approaches, (iv) ridge-based ap- proaches, (v) differential and mathematical geometry-based approaches and (vi) matching filters approaches.

The proposed method in this paper aims to develop a more accurate extraction of the blood vessels in each region of the tree with a view to enhance the detection accu- racy while still reducing the false alarm rates. To achieve this, we propose to modify and extend the MF approach by using multiscale match filters and by incorporating some local features. Unlike existing methods, it can be observed that a multiscale filter approach enhances the detection of small blood vessels, and by incorporating local features some confidence measures are added for a more accurate blood vessel detection. This paper is organized as follows: Section 2 gives a brief overview on the matched filter approach for retinal image segmentation. Section 3 introduces the pro- posed method while Section 4 describes the experiments carried out. The results are discussed in Section 5 including a comparative study against other methods. Finally, Section 6 concludes the paper.

Blood vessel extraction using MF approaches convolve the image with multiple matched filters Chaudhuri, Chatterjee, Katz, Nelson, & Goldbaum (1989). Designing matched filters with different orientations and sizes plays a crucial role in such methods. MFs are usually followed with some other image processing operations like thresholding to get the final vessel contours.The original work on matched filters was proposed by Chaudhuri et al. Chaudhuri et al. (1989), in which they introduced matched filters to enhance blood vessel in the image and suggested using a proper thresholding scheme to distinguish between vessel and background; in their work they have used the Otsu method Otsu (1979) for thresholding.Hoover et al. Hoover, Kouznetsova, & Goldbaum (2000) combine local and region- based properties to segment blood vessels in retinal images. The method thresholds the matched filter response (MFR) Chaudhuri et al. (1989) using a probing technique. Classifying pixels in an area of the MFR as vessels and non-vessels is done by iteratively decreasing the threshold. At each iteration, the probe examines the region-based attributes of the pixels in the tested area and segments the pixels classified as vessels. Pixels that are not classified as vessels from probes are recycled for further probing. Hoover et al. have collected a database of 20 manually labeled images (STARE database), which is publicly available together with the results of their method.

Figure 1: Profile of a cross section of a blood vessel



Cinsdikici et al.(2009) proposed a (MF/ant algorithm) method as a hybrid model of matched filter and ant-based clustering aiming to im- prove the accuracy and false/true ratios performance of blood vessel detec- tion. They have tested their algorithm on 20 images from the DRIVE database Staal, et al (2004). Zhang et al.(2010), proposed combining the matched filter with first-order derivative of the Gaussian (MF-FDOG), as an extension and generalization of the MF. They used first-order derivative of Gaussian in order to reduce the problem of strong response of Matched Filter in non-vessel edges which could achieve much higher vessel detection accuracy than the MF alone. This method was tested on 20 images from each of STARE and DRIVE databases Hoover et al. (2000); Staal et al. (2004).

Dalmau et al. Dalmau & Alarcon (2011), worked on enhancing detection accuracy by working on the thresholding technique. Instead of using a threshold directly, they relax an automatically obtained threshold value in order to obtain two thresholds: one for the object (vessels) and the other for the background (non-vessels). After applying both thresholds, they use a Cellular Automata Vezhnevets & Konouchine (2005) as segmentation method. They have tested their method on both STARE and DRIVE databases Hoover et al. (2000); Staal et al. (2004) with 20 images from each. Staal et al. (2004), and Jiang and Jiang & Mojon (2003) are well-known methods which are not based on matched filters, but related to our work in a sense that they are all considered as rule-based. Staal et al. (2004), worked on

extraction of image ridges, which coincide approximately with vessel centerlines. The ridges are used to compose primitives in the form of line elements, an image is partitioned into patches by assigning each image pixel to the closest line element.

Every line element constitutes a local coordinate frame for its corresponding patch. For every pixel, feature vectors are computed that make use of properties of the patches and the line elements. The feature vectors are classified using a KNN-classifier and sequential forward feature selection. For testing their method on a large database, they have constructed the DRIVE database of 40 manually labeled images, divided into two sets for training and testing. They also tested their method on STARE database Hoover et al. (2000). Their database is publicly available (DRIVE database), and has been widely used for experiments. Jiang and Mojon (2003) proposed a general framework of adaptive local thresholding based on a verification-based multi-threshold probing scheme. Object hypotheses were generated by binarization using hypothetic thresholds and accepted/rejected by a verification procedure. Their proposed algorithm was applied on blood vessel extraction; Their proposed method shared with Hoover et al. who used threshold probing. The method was tested on DRIVE database Hoover et al. (2000) had compared results to global thresholding.

Retinal Segmentation Using Matched Filters (MF): MF was first proposed by Chaudhuri et al (1989) to detect vessels in retinal images. It was based on the assumption that the cross-section of the vessels can be approximated by a Gaussian function. A vessel segment and the local gray level distribution is shown in Fig. 1. One can see that the vessel sectional profile can be assumed as a Gaussian shape and the proposed 2D Gaussian kernel is suitable when applied to local blood vessels since the vessels may be considered as piecewise linear segments. Instead of matching a single intensity profile of the cross section of a vessel 1D, an improvement can be achieved by matching a number of cross sections (of identical profiles) along its length simultaneously with a 2D matched filter Chaudhuri et al. (1989).In MF approach, multiple 2D Gaussian matched filters with different orientations are convolved with the given retinal image and the highest responses of the directional filters are then threshold in order for the blood vessel network to be extracted. Gaussian MF is defined as Chaudhuri et al. (1989); Lei Zhang & Zhang (2009):

$$g(x,y) = -\exp\frac{-x^2}{\sigma^2}, for\ x \geq 3\sigma, y \geq \frac{L}{2} \quad (1)$$

where L is the length of the segment for which the vessel is assumed to have a fixed orientation, and σ defines the spread of the intensity profile and represents the scale of the MF filter; the negative sign indicates that the vessels are darker than the background. Actually, a 2D MF is a 1D Gaussian function in x-direction and repeated in y-direction. For the detection of blood vessels at different orientations, the kernel has to be rotated accordingly, and the direction of the vessel is assumed to be aligned along the y-axis. For implementation purposes, g(x, y) is rotated to detect the vessels from different orientations. The rotation of g(x, y) with angle θ is Lei Zhang & Zhang (2009).

MATERIAL AND METHODS

As mentioned previously, an MF makes use of the prior knowledge that the cross- section of the blood vessels, which is a feature of the vessels, can be approximated by a Gaussian function. However, a well-known problem of this approach is that it responds not only to vessels but also to non-vessel edges such as edges of bright blobs and red lesions in the retinal images. In this paper, we propose an extension to the MF approach to reduce/ suppress false detections caused by MF. Our approach enhances the MF approach by addressing two main issues: (i) to be able to detect thin blood vessels and (ii) to reduce the false detection rates of non-vessel edges. To achieve these goals, we propose to employ a multiscale matched filter approach and by incorporating into the filter response some local features of the circular area formed by a wheel centered around each pixel in the retinal image.These features are then combined/fused to form a feature image which can then be threshhold to extract the blood vessel network.

Selecting the threshold value is a very critical issue. As mentioned earlier, the MF produces strong responses to vessels and edges non-vessel structures in the retinal images. As a result, selecting one global threshold may cause false detections. Therefore, we chose to deploy the first order derivative of Gaussian (FoDoG) to select the threshold value based on the FoDoG response at each pixel. In order to build a set of Multiscale Matched Filters (MSMF) we compute a set of scales (σi) i=1,2, • • • ,m; a set of lengths (Li), i = 1, 2, • • • , m; one L value for each sigma; a set of orientations (θi), i = 1, 2, • • • , n ; the number of orientation is n and the total number of convolution kernels is equal to n×m. The parameter Li can be selected based on σi.

If σi is small then Li is relatively small and vice versa. The orientations can simply be defined as the number of orientations n or the angular resolution (180/n). According to previous works Dalmau & Alarcon (2011), 12 orientations (n=12) produce good experimental results, i.e., an angular resolution of 15o. The scales used in our proposed method for the MSMF for both databases are: σ [1, 1.5, 2] , L [7, 7, 9] and orientations n=12; resulting in 36 kernels.

For the LFs extension, we have used two local features obtained from the circular area around each pixel as follows:

i LF_1 : the MSMF response of the center pixel.
ii LF_2 : the difference between the MSMF value of center pixel and MSMFmin the minimum MSMF response among all pixels inside that circle with rk radius around it; LF2 is computed using the following equation:

$$LF_2(i, j) = |MSMF(i, j) - MSMFmin(i, j)| \quad (2)$$

$$MSMFmin(i, j) = \min \{MSMF(x, y) \forall p(x, y) \in Crcl(p(i, j), rk)\} \quad (3)$$

where $Crcl(p(i, j), r_k)$ is a circle of radius r_k centered at pixel p(i,j).

These features represent a measure of confidence of the vessel detection method through their fusion and will result in images with much enhanced detected blood vessels; the details of these features and this enhancement step will be described in subsequent subsections. Our methodology requires some preprocessing steps such as extracting the Region of Interest (ROI) which is the retinal fundus and generating the retinal mask. This step will be described in the next section. Another important preprocessing step relates to image enhancement to remove uneven illuminations and enhance low contrast image using an Adaptive Histogram Equalization (AHE).

Locating ROI of Retinal Fundus: A fundus image consists of a circular fundus and a dark background surrounding the fundus. It is important to separate the fundus from its background so that further processing is only performed for the fundus and not on non-useful pixels belonging to the background. In this sub-section a method for creating a binary fundus mask prior to lesion detection is described. In a fundus mask, pixels belonging to the fundus are marked with 1's and the background of the fundus with 0's. With the help of the fundus mask a lesion detection algorithm can process only the pixels of the fundus and omit the background pixels. The fundus can be easily separated from the background when the original fundus image has been converted from the RGB color system to any color system where a separate channel is used to represent the intensity values of the image Kuivalainen (2005) such as HSI, which we have chosen for our work in this paper.

The intensity channel can be threshold by a low threshold since the background pixels are typically significantly darker than the fundus pixels. The threshold value can be fixed to certain low value for all images; in our work we used the Otsu method to determine a threshold for each image Otsu (1979).In some cases, the intensity difference between the fundus and the background is not very clear due to inadequate illuminations near to the edge of the fundus. Thus, some dark regions of the fundus may be considered as background after the thresholding process. However, very dark pixels near to the fundus edges can be excluded from further processing since it is not possible to detect lesions from regions where illuminations have been almost zero. After thresholding, a median filter is performed to remove single noise pixels from the created fundus mask. The edge of a fundus tends to be very noisy, and thus, the edge pixels are removed by a morphological erosion operation with a small structuring element (clearEdgeThickness) Kuivalainen (2005). The remaining holes in the mask can be filled by processing the mask line by a line and filling the missing pixels between the first and last fundus pixels in each row. An example of a created fundus mask is shown in Figure 2.

Figure 2: Sample of locating retinal fundus for image im0163 from STARE DB

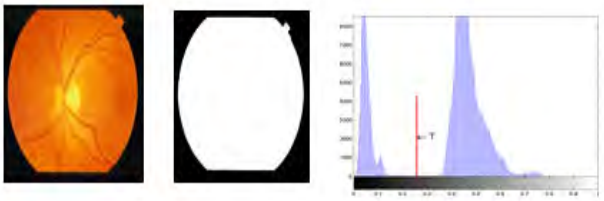
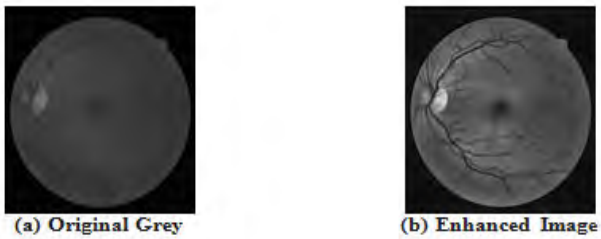


Figure 3: Sample of AHE enhancement to image 01 from DRIVE DB



Proposed Retinal Image Enhancement: Contrast enhancement techniques are used widely in image processing; and histogram equalization (HE) is one of the most popular automatic procedures Stark (2000); Wu, Zhang, Liu, & Bauman (2006). Although it is widely used, HE is less effective when the contrast characteristics vary across the image. Adaptive HE (AHE) Stark (2000) overcomes this drawback by generating the mapping for each pixel from the his- togram in a local window. AHE does not allow the degree of contrast enhancement to be regulated. Previous works have shown that AHE is more effective than the classical histogram equalization, especially when detecting small blood vessels characterized by low contrast levels. As the green channel of retinal images presents the largest contrast between the blood vessels and the background Dalmau & Alarcon (2011); Soares, Le, Cesar, Jelinek, & Cree (2006).

In our proposed method, we converted the retinal color images into gray scale images by keeping the green channel, and discarding the rest of the color channels. We then applied the AHE to enhance contrast, Fig. 3 shows an example of the enhancement on retinal image.

Proposed Method (MSMFLFs): As described at the beginning of this section, our proposed method extends the MF approach by employing a multiscale matched filter and incorporating some local fea- tures of the circular area formed by a wheel centered around each pixel in the retinal image. We also have deployed the first order derivative of Gaussian (FoDoG) to select an adaptive-local threshold value based on the FoDoG

response at each pixel. The following steps describe briefly the proposed process:

1. Convert images from RGB color space to HSI color space and determine the fundus mask.
2. Convert the green channel of RGB color space to gray level and enhance the contrast.
3. Create proposed multiscale MSMFs using multiple scales.
4. Convolve the enhanced image with MSMFs using different orientations by selecting the highest response directional filter response.
5. The MSMFLF feature image is obtained using the following steps:
6. For each pixel P_0 inside the retinal area in image q(x, y), use a wheel of radius rk around it to define a circular area around that pixel (see Fig. 4) and get local features (LF_1, LF_2) as described in section 4 with equation (2 , 3)
7. Create the feature image MSMFLFs(x,y) by fusing the 2 features together using the following equation:

$$MSMFLFs(x, y) = LF_1(x, y) + LF_2(x, y) \quad (4)$$

vi Create the MSFoDoG response by convolving the feature image MSMFLFs(x,y) with a multiscale 2D first derivative of Gaussian with scales: σ[1, 1.5, 2], L [7, 7, 9] and orientations n=12. Select the smallest response among the 36 kernels.

vii Using the MSFoDoG response to the feature image MSMFLFs(x,y), find a map of optimal threshold values TAdp, with a threshold for each pixel;

viii Threshold MSMFLFs(x, y) by TAdp to get the initial image of extracted vessel network v0(x, y) as:

$$v_0(x,y) = \begin{cases} 1\,(V); if\ MSMFLFs(x,y) \geq T_{Adp\,(x,y)} \\ 0\,(NV); if\ MSMFLFs(x,y) < T_{Adp\,(x,y)} \end{cases} \quad (5)$$

where T_{Adp} is the threshold map formed by the FoDoG map and feature image MSMFLFs, and each pixel has a threshold value adaptively set. The abbreviation (V, NV) stand for vessel and non-vessel.

ix Obtain the final vessel network v(x, y) by labeling objects in v (x, y) then removing objects that are smaller in size than Shr_L, where Shr_L is the shortest accepted length of a vessel segment;

Fig.(4) shows different cases of the processed pixels and our MSMFLFs method to detect the blood vessel pixels. In our proposed method, we used a wheel of radius rk around each pixel (see Fig. 5).

This wheel defines a circular area around the pixel of interest; and is used to collect the local features (LF1, LF2) as described in section 4 with equation (3, 4); these local features are used to form the final MSMFRLFs image with equation ((6)), which is then thresholded to get the segmented image. Fig. 6 shows an example to illustrate the enhancement process to improve the vessel detection and reduce false detections by using our MSFoDoG technique to adaptively estimate a threshold map. As can clearly be seen in the original image of Fig. 6(a), there are bright lesions. Fig. 6(b) shows the MSMFLFs feature map, in Fig. 6(c) we can see that with a global threshold value (e.g., Otsu method) these lesions may get falsely detected as blood vessels. However, in Fig. 6(d), which illustrates our MSFoDottLm method, the local mean map of the response of MSFoDoG clearly shows that it has high value in areas of bright lesions and low values for vessel areas; this feature is used to adaptively increase the threshold value in these areas which will help in reducing false detections; Fig. 6(e) shows the result of our proposed method, compared to ground truth image shown in Fig. 6(f).

Figure 4: Illustration of effect of using local features on vessel detection accuracy (4 cases): circular area in (yellow) color is the wheel defining the circular area around center pixel Po, in a snapshot of the image area and showing values of local features.

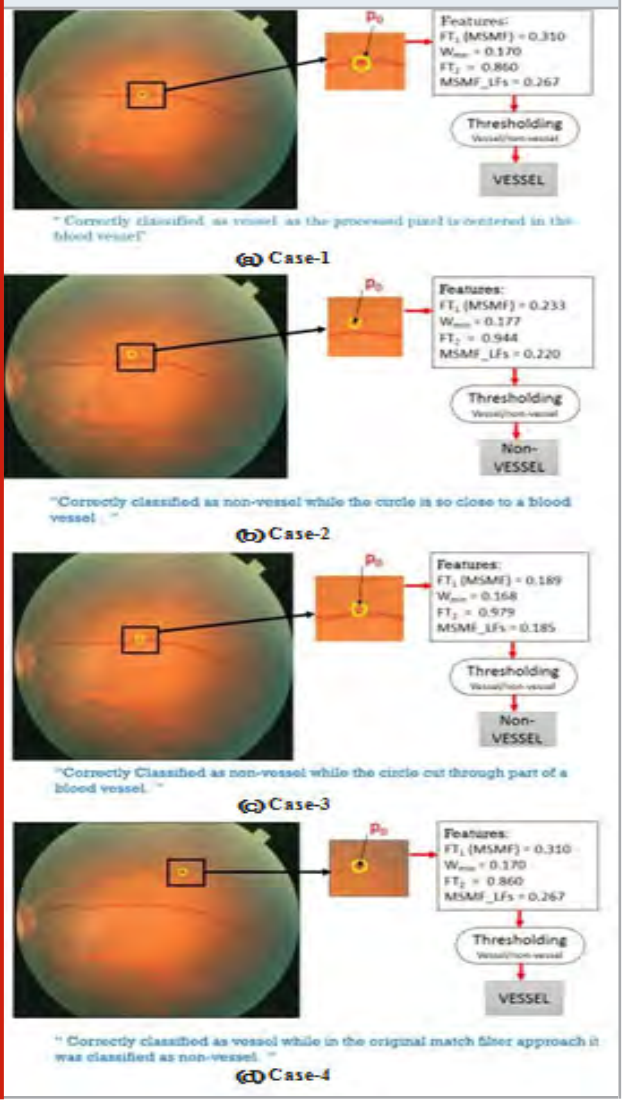
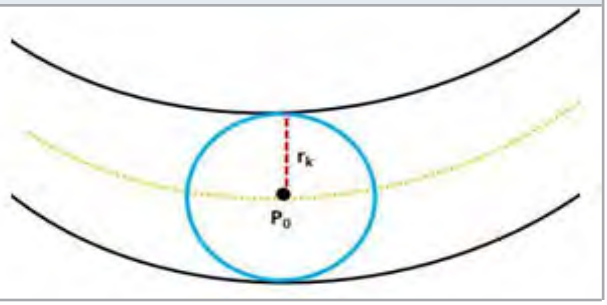


Figure 5: Circular area: In (blue) color is the wheel defining the circular area around center pixel Po , rk is the radius of the wheel.



RESULTS AND DISCUSSION

The proposed algorithm has been evaluated on real retinal images from the publicly available STARE Hoover et al. (2000) and DRIVE Staal et al. (2004) databases. The STARE (Structured Analysis of Retina) database contains twenty retinal fundus slides and their ground truth images. The digitized slides are captured by a TopCon TRV- 50 fundus camera with 35 degree field of view. Each slide was digitized to produce a 605x700 pixel image. All the twenty images were carefully labeled by hand to produce ground truth vessel segmentation by an expert.

Table 1. Methods used for comparison

Method	Methodology	Databases
Chaudhuri et al. (1989)	General matched filters (MF). Implemented by later works for comparison reasons e.g Cinsdikici & Aydin (2009); Zhang et al. (2010);	STARE (20 images). DRIVE (20 images).
Hoover et al. (2000)	Based on matched filters with threshold probing technique	STARE (20 images).
Cinsdikici & Aydi (2009) Zhang et al. (2010)	Used a (MF/ant algorithm) Used (MF-FDOG)	DRIVE (20 images). STARE (20 images). DRIVE (20 images).
Dalmau &Alarcon (2011)	Based on a relaxed thresholding technique with a Cellular Automata segmentation method.	STARE (20 images). DRIVE (20 images).
Staal et al. (2004)	Ridge-based method	STARE (19 images). DRIVE (20 images).
Jiang & Mojon (2003)	Using adaptive Local thresholding based on a verification-based multi-threshold probing scheme	DRIVE (20 images).

The DRIVE (Digital Retinal Images for Vessel Extraction) database consists of 40 color fundus photographs whose images are digitized using a Cannon CR5 non-mydriatic 3CCD camera with a 45 degree field of view. Each image is captured using 24-bits per pixel at the image size of 565584. In our experiments, we have used 20 images from each of the two databases, STARE and DRIVE. In both databases, the original images are in color. The green channel of the color fundus images was used for vessel extraction.

Parameter Settings: There are three types of parameters to be set efficiently: parameters of the MSMF algorithm, local features extraction, and the FoDoG algorithm. Since it is hard to assign the best values to these parameters we have chosen an empirical approach based on knowledge about the type of data (i.e., blood vessel). For the MSMF algorithm one needs to set the values for (number of orientations (n), σ , L). In our experiments using the multiscale MF (MSMF), the scales used for both databases have been chosen as follows: $\Sigma = [1, 1.5, 2]$, $L = [7, 7, 9]$.

From previous works 12 orientations (n=12) have been used Dalmau & Alarcon (2011) resulting in 36 kernels; The same parameters are also used for the FoDoG algorithm for the purpose of comparison. Local feature extraction algorithm is a very important part of our proposed method. Here the features are extracted based on a wheel with a radius rk placed around each pixel in the image to capture information used to form the feature image. The widths of the vessels are found to lie within a range of 2-10 pixels Chaudhuri et al. (1989). Based on this finding from our experiments, we have set rk = 9.

Performance Measures: The measures used to quantitatively evaluate the performance of our algorithm and compare it to some similar techniques include:

Detection accuracy (ACC), True posi- tive rate (TPR) and False positive rate (FPR). In our experiments, we have evaluated the performance results of our proposed method on each of the DRIVE and STARE databases. This has been carried out as follows:

i. For each image, compute the evaluation matrices (ACC, TPR, FPR)
ii. Compute the average (ACC, TPR, FPR) of all images.

As mentioned earlier, the proposed algorithm has been assessed on real retinal images from the publicly available STARE Hoover et al. (2000) and DRIVE Staal et al. (2004) databases using twenty retinal fundus slides and their ground truth images from each database. It was compared with other methods, Table-1 shows a list of related methods, used methodology and the dataset used. Tables 2 and 3 show the results of our proposed method using DRIVE and STARE databases respectively. From these two tables, it can be seen that the mean TPR obtained is (0.7661%) for STARE and (0.6312 %) for DRIVE while the mean FPR is (0.0311 %) for STARE and (0.0183 %) for DRIVE. Therefore, the FPR does not exceed (0.0512%) for STARE and (0.0242 %) for DRIVE. Figures 7 to 10 show sample results of our method from both databases.

Table 2. Results on DRIVE DB

Image	ACC	TPR	FPR	TNR	FNR
1	93.45	0.6602	0.0228	0.9772	0.3398
2	93.61	0.6749	0.0167	0.9833	0.3251
3	91.77	0.5285	0.0133	0.9867	0.4715
4	93.37	0.5739	0.0086	0.9914	0.4261
5	93.44	0.6035	0.0112	0.9888	0.3965
6	92.78	0.5814	0.0131	0.9869	0.4186
7	92.90	0.6250	0.0230	0.9770	0.3750
8	92.37	0.5717	0.0242	0.9758	0.4283
9	93.82	0.6032	0.0152	0.9848	0.3968
10	94.23	0.6330	0.0138	0.9862	0.3670
11	93.42	0.6500	0.0224	0.9776	0.3500
12	93.54	0.6104	0.0163	0.9837	0.3896
13	92.82	0.6185	0.0188	0.9812	0.3815
14	93.86	0.6791	0.0257	0.9743	0.3209
15	94.09	0.6756	0.0275	0.9725	0.3244
16	93.46	0.6262	0.0170	0.9830	0.3738
17	93.39	0.5939	0.0166	0.9834	0.4061
18	94.50	0.6973	0.0215	0.9785	0.3027
19	95.10	0.7169	0.0155	0.9845	0.2831
20	94.69	0.7013	0.0225	0.9775	0.2987
Average	93.53	0.6312	0.0183	0.9817	0.3688

Table 3. Results on DRIVE DB

Image	ACC	TPR	FPR	TNR	FNR
im0001	94.30	0.7488	0.0333	0.9667	0.2512
im0002	93.53	0.6155	0.0316	0.9684	0.3845
im0003	94.58	0.7345	0.0354	0.9646	0.2655
im0004	94.89	0.6796	0.0201	0.9799	0.3204
im0005	93.75	0.6894	0.0267	0.9733	0.3106
im0044	93.87	0.8332	0.0512	0.9488	0.1668
im0077	94.89	0.8594	0.0402	0.9598	0.1406
im0081	95.32	0.8728	0.0376	0.9624	0.1272
im0082	94.87	0.8343	0.0375	0.9625	0.1657
im0139	93.73	0.7879	0.0440	0.9560	0.2121
im0162	94.37	0.7921	0.0380	0.9620	0.2079
im0163	95.11	0.8283	0.0341	0.9659	0.1717
im0235	94.82	0.7658	0.0264	0.9736	0.2342
im0236	94.95	0.7688	0.0242	0.9758	0.2312
im0239	95.17	0.7969	0.0277	0.9723	0.2031
im0240	93.67	0.6553	0.0166	0.9834	0.3447
im0255	95.26	0.7675	0.0210	0.9790	0.2325
im0291	97.10	0.7426	0.0114	0.9886	0.2574
im0319	96.18	0.8326	0.0302	0.9698	0.1674
im0324	94.15	0.7162	0.0351	0.9649	0.2838
Average	94.73	0.7661	0.0311	0.9689	0.2339

Table 4: Comparison of different methods on the DRIVE DB

Method	TPR	FPR	ACC
2nd Human observer	0.7761	0.0275	94.73
Staal et al. (2004)	0.7194	0.0227	94.42
MF Chaudhuri et al. (1989);	0.6168	0.0259	92.8
Jiang & Mojon (2003)	-	-	89.11
MF-FDOG Zhang et al. (2010)	0.7120	0.0276	93.82
Cinsdikici & Aydin (2009)	-	-	92.93
Our Method	0.6312	0.0183	93.53

Table 5: Comparison of different methods on the STARE DB

Method	TPR	FPR	ACC
2nd Human observer	0.8949	0.0610	93.54
Hoover et al. (2000);	0.6751	0.0433	92.67
Staal et al. (2004)	0.6970	0.0190	95.16
MF-FDOG Zhang et al. (2010)	0.6134	0.0245	**93.84**
MF-MET Dalmau & Alarcon (2011)	**0.7380**	**0.0604**	**91.84**
MFCA Dalmau & Alarcon (2011)	**0.7606**	**0.0599**	**92.12**
Our Method	**0.7661**	**0.0311**	**94.73**

For the DRIVE database, fig.8 shows a sample result of blood vessel extraction in image-19 and the evaluation of the extracted image shows that the accuracy of extraction compared to the ground truth image is (95.10 %) with very good true positive rate (0.7169 %) while at the same time maintaining a low false positive rate (0.0155 %). This shows that our method to some extent does not miss-

classify non-vessel objects as blood vessel. Fig.11 shows a scatter plot for the extraction accuracy values of our method on the 20 tested images from DRIVE database. The red line marks the level for mean accuracy, and the Y-axis is bounded by minimum (91.77 %) and maximum (95.10 %) accuracy values for our method. From this figure one can see that for nearly more than (50%) of the tested images the accuracy is above the mean value (93.53 %). In the case of the STARE database, a sample result of our method is shown in fig. 9 for image im0129 and the evaluation of extraction results compared against the ground truth image, has given an accuracy of (97.10 %) with a true positive rate (0.7426%) and a false positive rate (0.0114 %). These results suggest an excellent extraction accuracy. From the scatter plot for the extraction accuracy values of our method on the 20 tested images from STARE database are shown in Fig.12, where the Y-axis is bounded by minimum (93.53 %) and maximum (97.10 %) accuracy values for our method while the mean accuracy is marked by the red line. This figure clearly shows that more than (50%) of the tested images have an extraction accuracy higher than the mean value (93.53 %).

Figure 6: Illustration of the proposed MSMFLFs method for blood vessel extraction

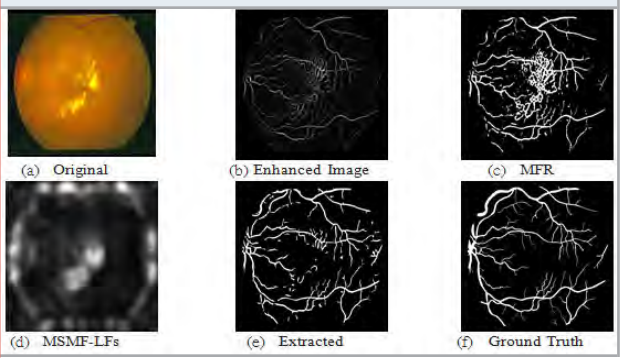


Figure 7: Results of image 02 from DRIVE DB; ACC=93.61, TPR=0.6749, FPR=0.0167

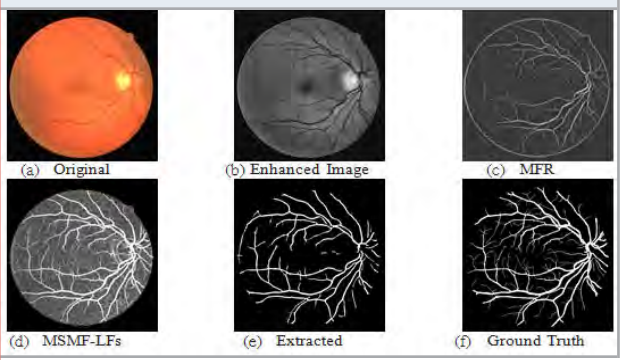


Figure 8: Results of image 19 from DRIVE DB; ACC=95.10, TPR=0.7169, FPR=0.0155

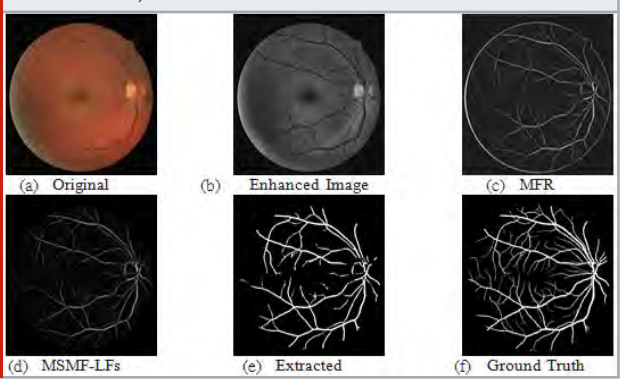


Figure 9: Results of image im0291 from STARE DB; ACC=97.10, TPR=0.7426, FPR=0.0114

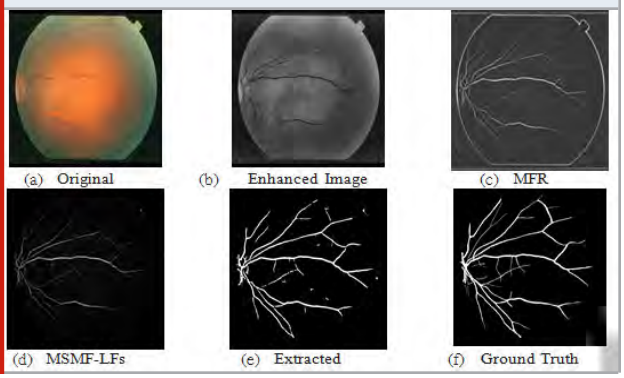


Figure 10: Results of image im0255 from STARE DB; ACC=95.26, TPR=0.7675, FPR=0.0210

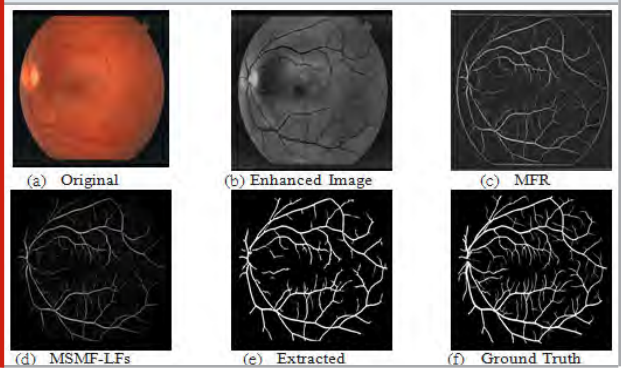


Figure 11: A scatter plot for accuracy values of our method on tested images from DRIVE database; The red line marks the level for mean accuracy; The Y-axis is bounded by minimum and maximum accuracy values for our method.

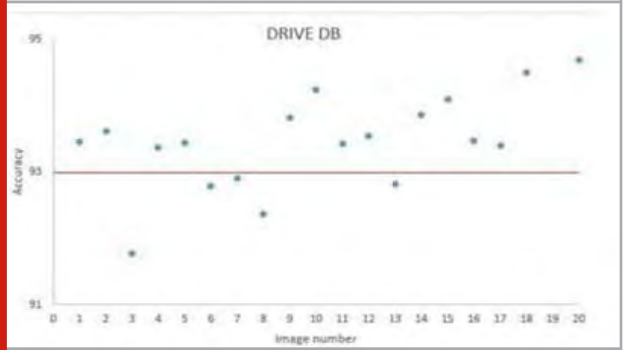
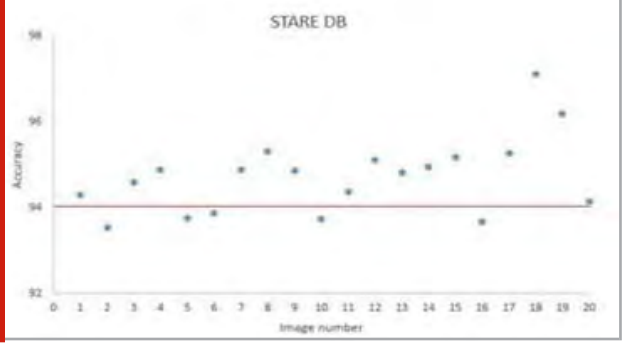


Figure 12: A scatter plot for accuracy values of our method on tested images from STARE database; The red line marks the level for mean accuracy; The Y-axis is bounded by minimum and maximum accuracy values for our method.



For the purpose of quantitatively evaluating the performance of our algorithm, a com- parative study against some similar methods in literature has been carried out. Tables 4 and 5 show the results of this comparison. It can clearly seen that our proposed method gave high ACC values. While in the cases of methods with higher ACC value than ours, we either have a higher TPR or a lower FPR.

CONCLUSION

In this paper we have presented a novel extension to the matched filter approach for use in blood vessel detection of retinal images. An adaptive thresholding scheme using FoDoG was developed and the proposed method (MSMFLFs) was evaluated on publicly available DRIVE and STARE databases. The results obtained are promising and compare favorably against those obtained using existing and similar methods. A possible enhancement to this method could be to deploy some other features and/or experimenting different thresholding techniques. Although, our method was proposed in the context of detecting blood vessels from retinal images an ongoing work is under- way to develop a quantitative method based on the shape and regularity of detected blood vessels in order to detect possible signs/symptoms of diseases.

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A Knowledge-Based System to Identify the Potential Blood Donors

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ABSTRACT

Blood donations help save millions of lives every year. According to the World Health Organization, almost 120 million blood units are collected each year to help people with various health conditions. But this still doesn't meet demands. Blood cannot be stored indefinitely, making blood unit collection a challenge. Furthermore, even though blood banks run blood donation campaigns regularly, some patients are suffering from the lack of suitable blood types in blood banks. Additionally, finding appropriate donors is another common challenge facing blood banks. In this paper, we have proposed a scheme to improve the performance of blood banks and increase the chance to find suitable blood donors promptly. Besides, our system helps to select an effective target group for blood donation campaigns. The proposed blood bank system is artificial intel-ligence-based; it depends on machine learning algorithms to enhance the efficiency of the process of finding potential blood donors. Additionally, the blood donor database is not limited to people who have provided their information to blood banks as anticipated blood donors. It also includes some people who have never visited blood banks. In the suggested system, a machine learning algorithm classifies people in the database into two groups: people who are more likely to donate their blood and those who are less likely to donate blood. The classification relies on the factors that affect a person's be-havior, such as the education level, work environment, culture, and personality. One added benefit of the system would be encouraging blood donation among previously reluctant blood donors.

KEY WORDS: MACHINE LEARNING, BLOOD BANK, CLASSIFICATION, BLOOD DONATION.

INTRODUCTION

Blood donation is the "act of giving blood" which can be used to save lives Harmon&t Angela (2019). Ac-cording to the American Red Cross, someone will need a blood transfusion every two seconds. Because the blood does not have a substitute, volunteer blood donations are important. Blood donation does not harm a healthy person; it typically is a short process that can help someone in need. One blood unit (450 - 500 ml) can help four people. Donated blood units have various uses some of them will be mentioned. Blood donation helps patients with cancer, thalassemia, sickle cell disease, and other diseases. It also helps a person who has lost blood due to accidents, surgeries such as organ transplants, heart, and women with complica-tions during child-birth Rahman et al. (2011), Arif et al. (2012), Nabil et al. (2020), and Das et al. (2020). As figure 1 shows, most cases need to donate, they are cancer patients, accident victims, sur-gery, medical uses for extract Plasma to treat some diseases,

ARTICLE INFORMATION

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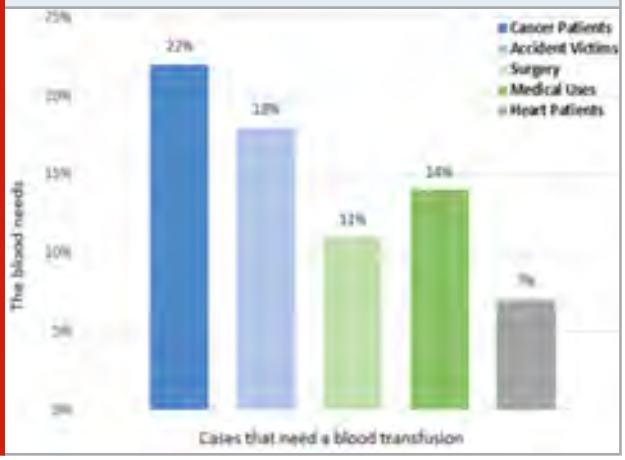
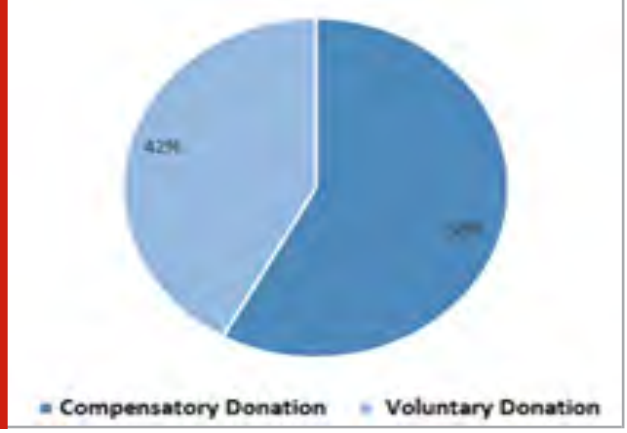
and heart patients to supply pa-tients of Coronary artery disease Wateen (2019) and Nabil et al. (2020).

Figure 1: Cases Need a Blood Donation



During the donation process, the person will give between 450 to 500 milliliter of blood from about 4,500 to 5,700 milliliter in his/her body Blood donation (2013). How much blood is in the human body (2017) and Naresh & Nagesh (2020). According to the Ministry of Health in Saudi Arabia, 90 million units of blood are donated each year globally Hematology - Blood Donation (2018). Regardless of that, the demand for blood transfusion is on the increase. In Saudi Arabia, only 42%of the blood banks' need is covered by voluntary donation and the remaining 58% is covered by a compensatory donation by donation from patients' relatives and friends while the goal is to reach self-sufficiency with 100% voluntary donations, see figure 2.

Figure 2: Blood Donation Types



Furthermore, according to the results of the survey that we have collected which is shown to us that 84.1% of the people who answer the survey never donated and only 15.9% have do-nated before. That means there is a lack of blood donation. The de-tails of this survey have been explained in section 5. Many organizations in Saudi Arabia that works with the ministry of health to increase voluntary blood donation percentage" such as Wateen "Wateen is a na-tional blood donation platform Harthy (2018) and World blood donor day (2016) and Das et al. (2020). However, the problem with most existing blood bank systems that it does not cover the blood banks' need by 100% of voluntary donations. These systems rely on patients who need blood to provide donors if the blood bank does not have enough blood units which can take a lot of time and effort. As figure 3 shows, a model of the existing blood bank system Harthy (2018).

Figure 3: Existing Blood Bank System

MATERIAL AND METHODS

The main goal of this proposal is to improve the blood bank system by reaching out to people more likely to donate through study some factors that affect their behavior by taking ad-vantage of the ML algorithms then contact them. That will help increase voluntary donation. This section is an overview of the concepts and definitions related to the proposed work. A brief description of the blood bank and machine learning is provided. Blood Bank is the cen-ter responsible for all blood-related operations where blood is collected from donors, blood tests and blood components donated, stored, treated and transported to patients in need of blood transfusion. Donated blood (whole blood) is sometimes separated, each component separately, and transferred to a different person as needed. The blood center may be inde-pendent or part of a hospital Obeagu et al (2016) and William (2018).

The history of blood bank starts at 1492 with the first blood transfusion attempt, then in 1901 was discovered blood groups A, B, and O. In 1907 was the first successful blood transfusion, and in 1932 has been created first blood store and transfusion center in the Middle East Wa-teen (2019), and now blood banks are everywhere. Saudi Arabia has over 260 World blood donor day (2016). Machine learning (ML) is "Field of study that gives computers the capabil-ity to learn without being explicitly programmed" ML what is machine learning? (2019). Ma-chine learning is done when machine learning algorithms are learned from the information directly, they did not dependent on predefined equation. Also, the improvement of algorithm performance depends on increasing the amount of sample available to learn What is machine learning (2019). The basic learn-ing models in ML are: Supervised Learning (SL), Un-supervised Learning, Semi-supervised Learning, and Reinforcement Learning Luo et al. (2020).

Several studies discuss blood bank systems and ways to improve their work. Most of them focus on providing non-knowledge-based blood bank systems that connect donors with recip-ients and blood banks. On the other hand, some focus on providing an effective blood bank system by using machine learning algorithm and classification techniques. The problem that we face in these studies was a few of them that use predictive in the blood donation sector. which made our reading and research limited in specific studies Naresh & Nagesh (2020).

Different ML algorithm have been used in researches that study ML algorithms and classifi-cation techniques in the blood bank sector. Some studies applied decision tree algorithms such as the CART algorithm was used in Santhanam & Sundaram (2010) to classify and identify blood donation behavior. The authors Ramachandran et al. (2011) used a decision tree (J48) algorithm to develop a system to analyze large datasets of donor blood groups. Also, the au-thors Zulfikar et al. (2018) used decision tree and Naive Bayes classifier to determine the eli-gibility of the donor by proposing a classification model that reduces time in the selection process and then compares their accuracy and performance which is naive bayes was better Luo et al. (2020).

Besides, some of them add deep learning algorithms to their studies such as, author Bahel et al. (2017) used artificial neural networks, decision tree (C5.0) and support vector machines to solve the problems on the performance of ML algorithms in existing studies that predict the appropriate donor by proposing a new prediction model. Also, authors Boonyanusith & Jittamai (2012) used artificial neural networks and decision tree (J48) to develop a classification model from different factors that influencing behaviors in blood donation and compare the results between algorithms.

Table 1. Review of Knowledge-Based Blood Bank Systems of Previous Studies.

Paper	Goal	Dataset	Algorithm Used	Accuracy
2010	Create a model that identifies blood donation behavior using classification algorithms.	Blood transfu-sion service center	Decision Tree (CART)	99%
2011	Identify an appropriate blood donor in a short time and high efficiency. By analyzing large datasets of donor blood groups	Database of (IRCS) Blood Bank Hospital.	Decision Tree (J48)	59%
2018	Reduce time in the selection process which defines the eligibility of the donor.	Not mentioned From the Blood	- Decision Tree - Naive Bayes	66.65% 79.95%
2017	Solve the problems on the performance of ML algorithms that predict the appropriate donor.	Transfusion Service Center in Hsin-Chu City in Taiwan.	- C5.0 -Artificial Neural Net-works - Support Vector Machines	88.37% 83.72% 76.74%
2012	Compare results in blood donor classification between ANN and DT.	An online ques-tionnaire at 4 universities in Thailand.	-Artificia Neural Net-works -Decision Tree (J48)	76.25% 75.75%
2009	Study what factors influence on blood donation behavior in Egypt and compare the classification performance of NNs against LDA.	Self-completion questionnaire to citizens in Port Said, Egypt.	- Multilayer Perceptrons - Probabilistic neural network - latent Drichlet allocation	98% 100%

The author Mostafa (2009) presented a detailed study of profiling or classifying blood dona-tion in Egypt. Where he lists four factors that influence blood donation. Which they are: altru-istic values, perceived risks, knowledge, and attitudes. Then he develops hypotheses to tease these factors on data that were collected in Port Said, Egypt using the drop-off, pick-up meth-od and compare two types of artificial neural networks models. This method is used in studies conducted in the Arab world due to the difficulty of reaching the respondents using mail questionnaires. In this study, the author listed only four factors that affect blood donation, but several factors are most influential in a person's actual coming to donate blood. In our proposed system, we will examine the factors mentioned by the author in his study Mostafa (2009) as well as other factors that are not

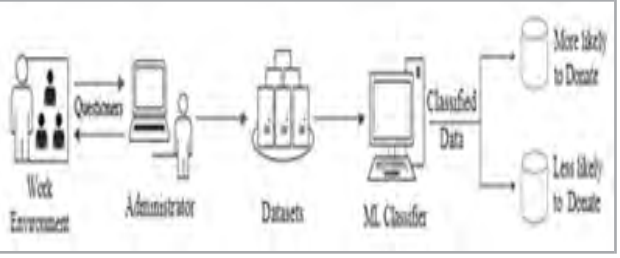
already mentioned and might have a greater impact on Saudi society such as Religious factors. Table 1 shows a summary of these studies.

The issues related to some of the previous studies was the predicted of the regulari-ty of donors based on the number of donation without considering the factors that may affect people in their coming to donate. However, some of them did not cover some important factors such as occupation and study major.

RESULTS AND DISCUSSION

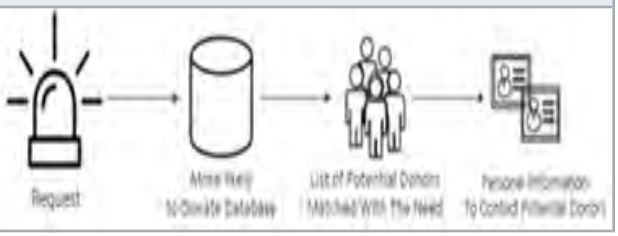
The big challenges facing blood donation in Saudi Arabia and globally are finding blood do-nors and encouraging non-donors to donate. This study presents a potential solution to some of those issues. Establishing an effective system of predicting who is more likely to donate blood would improve the probability of increasing blood donors. The proposed system will be done by developing a classifier using an ML algorithm that clas-sifies people into more likely to donate blood and less likely to donate based on some factors that affect people in responding to blood donation requests such as social, psychological, and religious. After developing the classifier, it will be able to predict the classification of new people through their data that have been collected from different sources such as universities, hospitals or other work environments. The proposed system model is shown in figure 4.

Figure 4: The Proposed System Framework



The process of developing the classifier requires providing previous data to the ML algorithm in the training phase. However, due to the lack of blood donor data sets that have the factors that affect the blood donors, we carried out a questionnaire to collect data the details of this survey will be explained in section 5. A closer look at the system's work and how it will help in real-life scenarios. First, the blood bank has to collect data from employees in different environments or students in universities via questionnaire. Then the system administrator will add that data to the system. After that, when the blood bank has a request for blood donation, the system will display the potential donors from the database, which contains in-formation of the people who are more likely to donate. Then, it will drop the records that do not match the request such as, people who have blood group does not match who in need to donate. Also, the system will automatically block the donor if it does not pass two months after the last donation. Figure 5 shows the utilization of the proposed system in real-life scenarios.

Figure 5: The Utilization of the Proposed System in Real-Life Scenarios



This system would be useful in different ways such as, devise strategies that target non-donors for example, through awareness campaigns on the importance of blood donation. Also, targeting donors through contacting them at urgent blood requests, and blood donation cam-paigns. The outcome of the proposed system will improve the efficiency of the blood bank system by finding blood donors more accurately, and reduce costs by contacting the potential donors rather than contacting someone how is not willing to donate. To implement the proposed system, we collected and analyzed data to train and test our mod-el.

Table 2. The First Section of the Second Questionnaire.

Question	Description of question
Age Gender Blood type	The age of the respondent. The gender of respondents. When a Respondent knows the type of blood group, he or she is often aware of the importance of donating, especially when it is a rare species
Education level	the level of education of respondent.
Work or study in a medical field	Because they work or study in a place where there is a need for urgent blood donation, and they may see patients in a serious condition in need of blood with the lack of suitable blood for them in the blood bank, so they are aware of the importance of donation.

Data Collection: There was not a data set which suits our system. Because of that, data col-lection is carried out using a Self-Administered Questionnaire (SAQ) which is a type of communication method. That is one of the approaches to collecting data through different channels. First Questionnaire: The first questionnaire

was conducted online to gain insight into the reality of blood donation in Saudi society. The survey contains general questions about the respondents such as, gender, blood unit, and if she/he has donated blood before. If the answer to the last question was affirmative, she/he will proceed to the second section of the survey, which includes several questions about the details of the respondent's experience in blood donation such as the reasons for blood donation. We collected 1,704 responses in this ques-tionnaire, of which only 271 (15.9%) had previously donated blood.

Second Questionnaire: The second questionnaire which is the one we will use to implement our model. This one had spread using both paper and online surveys to make sure that is reaching out to the largest possible segment of the community. The main objective of the sur-vey is to know the essential reasons that impulse people donate their blood. The survey con-tains two sections of questions. The first section contains personal questions about the re-spondents like age, gender, education level, and blood unit which is part of the factors that affect people's willingness to donate blood. The second section contains several questions about other factors that affect people's desire to donate, including psychological, health and other factors.

First questionnaire, first section contains general questions about the respondent

- Age: The age of the respondent.
- Gender: The gender of respondents.
- The type of blood: The blood type of respondent. This question helps us find out the most common blood type in Saudi Arabia, and the percentage of each type.
- Have you ever donated blood: When the respondent answers this question in the af-firmative, he will proceed to the second part of this questionnaire, which contains questions about the details of his blood donation experience.

Table 3. The Second Section of the Second Questionnaire.

Factor	Question	Description of question
personal	Are you regular in donating?	Donate approximately every three months if there is not a health issue prevents you. A regular respondent donation indicates an understanding of the importance of donation.
	Did you go through a health issue that made you need blood donation?	If the respondent has ever needed a blood donation, he/she will know the importance of donation.
	In most cases of blood donation was the reason for the donation?	The respondent will identify one of these options: volunteer, kinship or never before.
Social	Have you ever donated blood?	In the past, a respondent donation indicates an understanding of the importance of donation.
	Have you lost a relative or acquaintance because he/she needs blood?	If the respondent a harsh experience and lost some of his/her relative or acquaintance because of the lack of blood. This makes him/her aware of the importance of donation.
	Have you ever suffered in the search for a blood donor?	If the respondent had a harsh experience in the search for urgently suitable donors to save a human life makes him/her realize the importance of donation.

Cultural	Do frequent donations and social media engagement encourage you to donate?	We will examine the effect of this factor in encouraging people to donate blood.
	Did you know that people with chronic non-communicable diseases, except heart disease, are able to donate blood?	Knowing the respondent about the blood banks' need for donors make him/her aware of the seriousness of the matter and the importance of a donation.
	Did you know that blood expires after a period in the blood bank?	
	Did you know that the need for blood is not limited to injuries caused by car accidents?	
	Do you think that blood needs in Saudi Arabia are adequately covered and the blood banks does not need more donors?	
Health	Do you think that blood donation is similar to some health practices like cupping?	Because cupping is a solution to stimulate blood circulation and get rid of some blood, is the respondent think that donation is similar to cupping because it also activates the blood circulation.
Religious	Do you suffer from an infectious disease, take medicines or any other health excuse that prevents you from donating?	The respondent will not be able to donate blood if his/her suffer from some health excuse.
	Do you think the main motivation for your blood donation initiative is to save the lives of others?	Because it helps save the lives of others.
Misconcep-tions	Do you think that blood donation is a danger to the health of the donor like the transfer of infection or diseases?	As a result of the unawareness of some people on how to donate, so they refrain from donating for fear of transmission of diseases or infection.
	Do you think blood donation is a risk to women's health?	Due to the exposure to several health symptoms lose a quantity of blood during her life such as menstrual cycle and birth. Some people think that women should not donate.

Second section of first questionnaire, contains questions about donor's blood donation expe-rience.

- Reasons for your blood donation: This question determines why respondents previous-ly donated for personal or voluntary reasons or both of them.
- How many times did you donate blood: find out how many donations have been done by respondents.

Second questionnaire, the first section of the questionnaire contains some personal ques-tions, see table 2. After answering these questions, he/she will proceed to the second section of the question-naire, which contains several questions about some factors that affect people's desire to do-nate, including psychological, health, cultural, and other factors. These questions are divided

into several sections according to the factor that belongs to, see table 3.

CONCLUSION

In this study, we have discussed the need for more efficient blood bank systems that helps in-crease the numbers of blood donors. We have proposed a knowledge-based system for blood banks that increase the chance to find potential blood donors. The system will be classifying people into potential donors and people who may not donate their blood. The classification will be based on the factors that affect people's behaviors in responding to blood donation requests like people's values, and their culture. The system would not only improve the effi-ciency of the blood bank system, but it would also reduce costs by contacting potential donors rather than contacting someone how is not willing to donate. As future work, we will develop the proposed system using machine learning techniques.

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Mini Review on Strigolactones: Newly Discovered Plant Hormones

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ABSTRACT

Plant growth and development are dependent upon complex biochemical reactions where phyto-hormones are key players. Recent scientific developments in the research field of crop physiology have identified a class of novel plant hormones called strigolactones (SLs). Strigol, the first strigolactones was isolated in 1966. After twenty years of this discovery, the structure of strigol was completely elucidated. The significance of strigolactones lies in the fact that they act as rhizosphere signalling molecules, play important role in regulation of plant architecture, promote germination of root parasitic weeds which have fatal effects on plant growth. Strigolactones are play significant roles in plant biotic and abiotic stress responses. They have emerged as important biological targets to study different signalling pathways, stress responses and developmental stages of plant. Presently, two naturally occurring SL families have been reported. One of those is having stereochemical configuration of (+)-strigol and the other is having (–)-orobanchol. The most prominent role of SLs has found to help in seed germination in the Orobanche and Striga, parasitic weeds. They proved to be important bioactive compounds in in-planta and ex-planta signalling pathways and molecular botany. The potential uses of SLs in controlling parasitic weeds seed in agriculture, amplification of the branching in arbuscular mycorrhizal (AM) fungi is discussed in this review along with the biosynthesis, mode of action and roles of synthetic SL mimics in sustainable agriculture are highlighted. The objective of this work is to harness the benefits of SLs for sustainable agriculture in the near future. There are about 285 free full text out which 98 review articles are archived in PubMed database in the last five years, i.e, 2015-20, with the keyword “strigolactone”..

KEY WORDS: STRIGOLACTONES (SLS), PLANT HORMONE, STEREOCHEMISTRY, PARASITIC WEEDS.

ARTICLE INFORMATION

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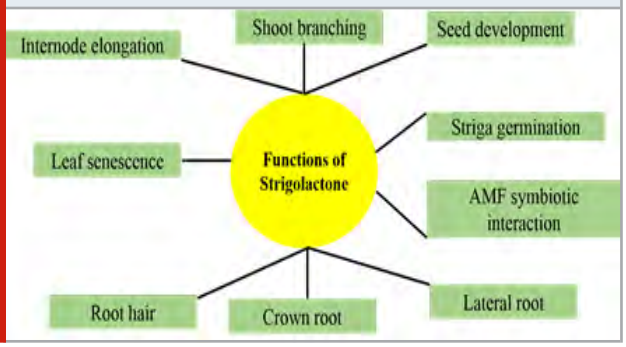
INTRODUCTION

In plant kingdom, the physiological features of plants are greatly affected by five phytohormones, namely, cytokinins (CK), auxins (IAA), abscisic acid (ABA), gibberellins (GA), and the aging hormone ethylene. The found hormones regulate different physiological functions; often one hormone is involved in controlling of several mechanisms in plants. Auxins are known to be primarily involved in stimulation of plant growth; cytokinins play role in regulation of cell division, making new plant organs; gibberellins (GAs) regulates stem elongation; ABA's major function is to regulate moisture; and ethylene plays important role in ripening of fruits and rotting. Phytohormones are interrelated in a complex manner and a number of their mechanisms are yet to be understood. A new class of phytohormones have been discovered recently, known as strigolactones (SLs). This class of phytohormones are involving in several biological processes that includes initiation of plant-fungal symbiosis, triggering of germination of parasitic plants that pose a major threat to farming (Bürger and Chory, 2020).

The rationale of writing this review article is as follows: Strigolactones (SLs) are most recently discovered plant hormones that has potential application in agriculture.SL biosynthesis takes place via carlactone (CL) intermediate and can serve as important target to study plant signalling pathways, stress responses and architectural development in molecular level. SL has been found to help in seed germination in the Orobanche and Striga, parasitic weeds, in regulation of the branching activity in AM fungi and also in control of plant architecture, which helps in sustainable agriculture. Of the various functions of strigolactones,most important ones are, that they can stimulate branching in plants, growth of symbiotic arbuscular mycorrhizal fungi (AMF) in the soil, impede shoot branching and trigger the germination of parasitic plant seeds as depicted in Figure 1.

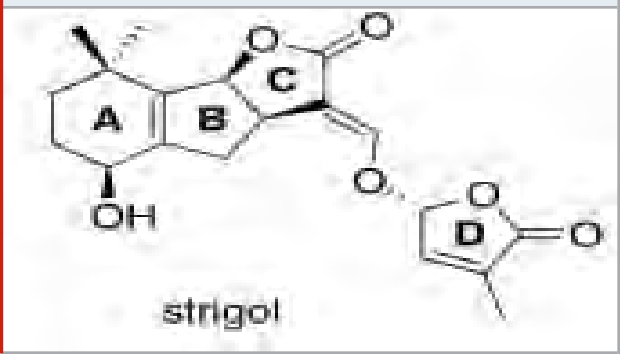
Figure 1: Functions of Strigolactones



Strigolactones performs a pivotal role in controlling plant growth and the developmental phases. This hormone has stirred curiosity among the scientists that is reflected in a good number of research publication published so far. In this article, recent developments in SL research has been described. Strigolactone was first identified by Cook et al., 1966 as (+)-strigol has been isolated from cotton (root exudates). It took almost twenty years to elucidate its full structural information (Cook et al., 1972; Brooks et al., 1985). One of the most important function of strigol is to help in germination of the seeds of Orobanche and *Striga* spp., these are the most common parasitic weeds found in agricultural fields. Before 1990, only one naturally occurring SL was known to the scientific community, that is, strigol. Novel SLs started to come in lime light from the year 1990.

They were being isolated from various natural resources. For an example sorgolactone has been isolated from the root exudates of sorghum (Hauck et al., 1992), in red clover, orobanchol has found (Yokota et al., 1998) and in tobacco, solanacol has found (Xie et al., 2007). Strigolactones were found in negligible quantity in root exudates, thus, their structural elucidation was very difficult; especially determining their stereochemistry was troublesome (Zwanenburg and Pospíšil, 2013). All SLs have the basic structural features, containing 3 annular rings, an ABC scaffold that is connected to a butenolide ring via an enol ether unit forming ABC-D structure as shown in Figure 1 (Zwanenburg and Ania, 2018). There are from the known SLs families, namely strigol and orobanchol. These two families differ in stereochemistry of B-C junction as in (+)-strigol and (–)-orobanchol. At C-2′ position, the spatial configuration of the butenolide D-ring in each naturally occurring SLs always remains the same (Figure 2).

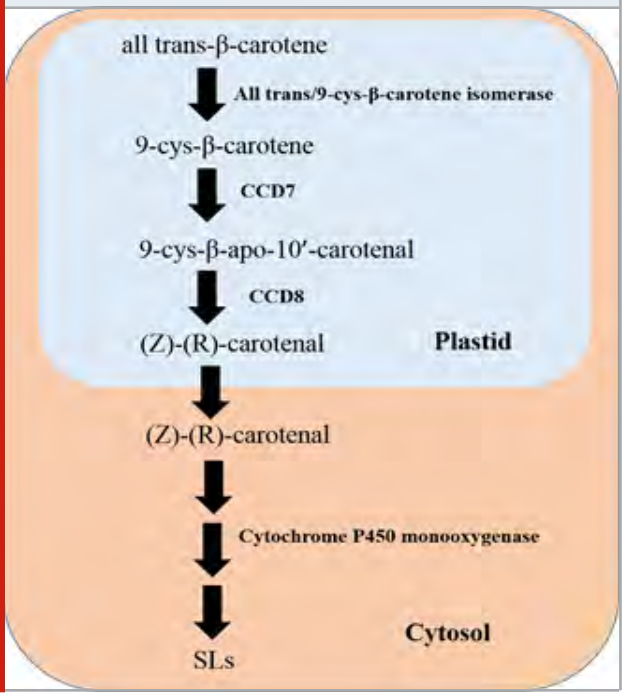
Figure 2: Basic structure of Strigol



Biosynthesis of Strigolactones (SLs): Strigolactones derive from carotenoids via two carotenoid cleavage dioxygenases CCD7 and CCD8 (Aly et al., 2014). Biosynthesis of SLs take place via a carlactone (CL) intermediate. Carlactone is formed from C40 all-trans-β-carotene by the enzymatic complex isomerization (β-carotene isomerase D27, chloroplastic) and oxidation reactions of the two carotenoid cleavage dioxygenases (Seto and Yamaguchi, 2014; Al-Babili and Bouwmeester, 2015; Flematti et al., 2016). Biochemical steps involving SL biosynthesis occurs in plastid of a plant cell and the product, i.e., CL is being exported to the cytosol as described in Figure 3 (Mishra et al., 2017). Carotenoid is the precursor of the central intermediate compound carlactone, and the stereospecific enzymes, viz., all-trans/9-cis-β-carotene isomerase (D27), the 9-cis-specific CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), CCD8 play are the major players (Jia et al., 2019).

Once the CL is exported in cytoplasm, it undergoes oxidation, closure of ring and functionalization of the involvement members from the family of CYP711 (Cytochrome P450 monooxygenase MAX1) (Zhang et al., 2014), and ultimately results in formation of SLs and SL-like compounds. It was reported that the enzyme cytochrome P450 monooxygenase or MAX1 in Arabidopsis thaliana was able to convert CL to carlactonoic acid, which in turn, again transform into a compound like SL called methyl carlactonoate (MeCLA) by an unknown enzymatic reaction (Abe et al., 2014). Once synthesized, these composites are transported through the plant and transferred into the rhizosphere. PhPDR1, a member of ABC family, is regarded as the key SL transporter (Kretzschmar et al., 2012; Sasse et al., 2015). Biosynthesis of SL occurs mostly in roots (sometimes in stems) is highly synchronised (Al-Babili and Bouwmeester, 2015). Starvation of Phosphate is reported to be strongly inducing the biosynthesis of SL (López-Ráez et al., 2008). The mutation study of SL-deficient and SL-insensitive has found in different plant species show that there must be a feedback mechanism of biosynthesis of SL regulation (Hayward et al., 2009; Proust et al., 2011).

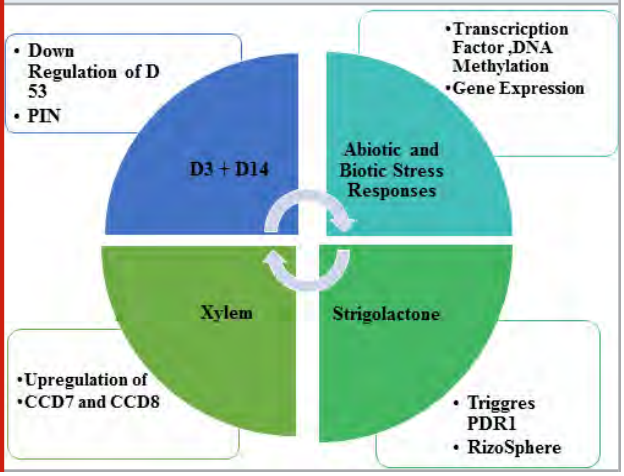
Figure 3: Biosynthetic Pathway of Strigolactones (SLs), Adapted from Mishra et al., 2017 Biochemical and genetic modulation of the SL pathway is identified as a promising approach to modify plant architecture, although whether and how the genes involved in the SL pathway play role in breeding still remain elusive (Wang et al., 2020).



Mode of Action Strigolactones: The mechanism of strigolactones action depends on the detachment of the D-ring by an enzyme forming a hydroxyl butenolide structure which is capable of inducing a change in conformation in a receptor pocket which will be initiating a biochemical cascade of reactions, reflecting in a signal transduction pathway (Zwanenburg et al., 2016). The first step of SLs activity is its interaction with a protein receptor called strigolactone esterase or D14 protein (Hamiaux et al., 2012; Kagiyama et al., 2013). An underlying molecular mechanism of germination process requires involvement of a part of active protein is called α/β-type hydrolase. This hydrolase contains a well-established catalytic chain of serine–histidine–aspartate having a big cone-shaped internal cavity that can encapsulate a SL molecule to accomplish the detachment of a hydroxy D-ring. It was reported that SLs change the phenotypic yield of PIN-FORMED (PIN) protein family of auxin transporters for calibrating of development and formative reactions (Hýlová et al., 2019; Lee et al., 2020; Zhang et al., 2020).

PIN genes are present in the genomes of multicellular plants exclusively and regulates asymmetric auxin distribution in multiple developmental processes that includes embryogenesis, organogenesis, tissue differentiation and tropic responses. Signalling pathway of SLs in rice is described in Figure 4. The signalling components in rice include a putative α β hydrolase receptor (D14), F-box protein, a component of SCF complex (D3), and a ClpATPase (D53). This complex is known to regulate the gene expression by modulating the degradation of various transcription factors, which act as either repressors or activators of transcription. Strigolactone distribution is regulated via PDR1 transporter within the plants and outside into the rhizosphere. PDR1 encodes Pdr1p (Pleiotropic Drug Resistance), a transcription factor involved in general drug response.

Figure 4: Signaling pathway of strigolactone



Mimics of Strigolactones: A class of compounds which lacks ABC scaffold but are capable to stimulate germination are known as SL mimics. As the organic synthesis of natural SLs is strenuous, there is an acute need for easy-to-synthesize and efficient analogs of SLs. These mimics perform the same functional activity like naturally occurring SLs, but have not exhibited the typically featured structured SLs. One of this group of mimics has an aryloxy substituent at C-5 and are called

debranones (branching furanones) and inhibits shoot branching (Fukui et al., 2013). *Strigahermonthica* seeds have high to medium response to debranones, whereas, parachlorophenoxy-debranone has the highest activity. Another finding says that there is a group of SL mimics are having an aroyloxy substituent at C-5 position of the D-ring (Zwanenburg et al., 2013) and they are modest germination stimulant for *S. hermonthica* seeds but are hyper active in *Orobanchecernua* and *Pelipancheramosa* seeds (Zwanenburg et al., 2016). Recently, Jamil et al., (2019) have synthesized and studied the biological activity of the SL analogs MP13 and MP26. They reported that methylation at C-3′ position in D-Ring of Strigolactone analogs reduces biological activity in root parasitic plants and rice. There is a lot of scope of research in the area of SL mimics to study their mechanism of action more clearly.

Applications of Strigolactones in Agriculture: Strigolactones play important role in molecular mechanism of plants including regulation of protonema branching, quorum which acts like signalling of a sensor in the moss *Physcomitrella patens* (Protust et al., 2011), factors affecting the branching form AM fungi, controlling parasitic weeds as well as plant architecture, etc. Some of the important applications of SLs are described below.

a) Strigolactones, a factor, helping in branching for arbuscular mycorrhizal (AM) fungi: SLs are reported to be the branching factors for AM fungi (Parniske, 2008). Orobanchol exhibits the highest activity, next comes 5-deoxystrigol. Like strigol, strigolactone analog GR24 is remarkably active but the mirror image of GR24 is ten thousand times less active. Another strigolactone analog GR7 (lacking A-ring) was found to be one thousand times less active than GR24. From the previous information it can be deduced that to act as an active branching factor for fungi, SL should have all the rings of the ABC scaffold. The stereochemistry too must be at par with the strigol family. However, it was reported that that the B-ring is not mandatory for branching factors.

The parasitic plants have symbiotic relationship with AM fungi (Bonfante and Requena, 2011) and the fungi behaves as natural soil fertiliser by expediting the mineral (phosphates, nitrates) uptake from soil. Phytohormones, microRNAs, secreted peptides regulate the development of arbuscular mycorrhizal (AM) symbiosis with the phosphorous status of the plant (Müller and Harrison, 2019). *Rhizophagus* triggers strigolactone biosynthesis gene expression in Arabidopsis roots and in the early stages of interaction, *Rhizophagus* activates the strigolactone biosynthesis genes CCD 7 and CCD 8 (Fernandez et al., 2019). In depth understanding of this type of symbiotic relationship may pave the path for newer experimental plans for controlling and managing the symbiosis between beneficial fungi and the parasitic weeds in agricultural lands. In depth understanding of this type of symbiotic relationship may pave the path for newer experimental plans for controlling and managing the symbiosis between beneficial fungi and the parasitic weeds in agricultural lands.

b) Strigolactones are inhibitors of shoot branching and control plant architecture: Strigolactones (SLs) are butenolide molecules that play essential roles in plant growth and development (Jamil et al., 2019). SLs crosstalk with other plant hormones in a cascade of events, though details of these interaction are still not clearly understood. Like all other phytohormones, the biosynthesis and action of SLs are controlled by other hormones. For example, cytokinins antagonizes the function of Sls in the outgrowth of axillary bud regulation (Dun et al., 2011) and mesocotyl elongation in dark (Hu et al., 2014). Similarly, auxins are key regulators of biosynthesis of SL (Hayward et al., 2009; Al- Babili and Bouwmeester, 2015). Auxins also found to be an antagonists of enhancement of auxin transport by SLs (Cheng et al., 2013). Lopez-Raez et al. (2010) demonstrated that abscisic acid, one of the important hormones took part in plant abiotic stress, finds importance in SL biosynthesic pathway. Vice-versa, SLs also regulate the abscisic acid biosynthesis (Al-Babili and Bouwmeester, 2015). Moreover, it was also reported other than hormones, presence of phosphates is inversely related with SL biosynthesis (Koltai, 2015).

Plant architecture control studies have been carried out with increased branching mutants, mainly with ramosus (rms) in garden pea has found decreased apical dominance (dad) in *Petunia hybrid*, more axillary growth (max) in Arabidopsis, dwarf (d) and high tillering dwarf (htd) in rice. Treatment with exogenous SL analog GR24 resulted in:

- inhibiting branching of shoots (Dun et al., 2013)
- stimulating growth of internode (Germain et al., 2013)
- speeding up of leaf senescence (Yamada et al., 2014)
- upregulating root hair elongation and growth of primary roots (Kapulnik et al., 2011)
- inhibiting outgrowth of axillary buds (Minakuchi et al., 2010)
- inhibiting formation of adventitious and lateral roots (Rasmussen et al., 2013)
- inducing stem thickness and secondary growth (Agusti et al., 2011) and other morphological changes.

c) Strigolactones control parasitic weeds: Parasitic weeds are responsible for huge crop losses and potential threat to agricultural production in all developing countries like India, Africa, and Middle East. The parasitic weeds seeds can be in inactive condition inside the soil for long period of time (several years of dormancy has been reported). They are activated by strigolactones secreted by any plant in vicinity that acts as host for the parasitic weeds (Parker, 2013). It is difficult to get control over these parasitic weeds in an eco-friendly way. Manual weeding is a tough job and least effective if already the parasitic weed has been invaded the host root system, exploiting

the necessary minerals and water. The crop rotation system and application of herbicides are sometimes not the best choice in under-developed or developing countries due to lack of modernized agricultural methods.

"Suicidal germination" is frequently used to combat with the situation. This approach relies on applying synthetic stimulant to the soil that can enhance germination in absence of host plant. Germination of parasitic weed will take place, but the seed will face untimely death due to the scarcity of nutrients. Now, the seedling of the crop are safely planted which can avoid the noxious parasitic weed. Suicidal germination approach was first tested in Hyderabad, India using GR5 to control the Strigahermonthica in sorghum (Johnson et al., 1976). It should be noted that the accumulation of synthetic analog of SLs in soil must be prohibited. A promising data of Orobanche ramose L. (hemp broomrape) in tobacco was obtained in field trials using Nijmegen-1 as a synthetic analog of SLs, along with a suitable emulsifying agent (Zwanenburg et al., 2016a).

Dissociation of the synthetic stimulant before commencing its action (Kannan et al., 2015) is an acceptable alternative to suicidal germination. Such type of decomposition is obtained in high alkalisoil by using borax (pH 9.2) or thiourea. Both borax and thioureaare nucleophiles that can separate the HO–D-ring. The parasitic weed seeds' germination by tomato seedlings was prohibited by the eco-friendly chemicals thiourea or borax application in the soil. Besides being a nucleophile, thiourea is an excellent antioxidant and thus, can readily deactivate reactive oxygen species required for the attachment of radicle of the germinated seed to the host plant's root system. Both the parasitic weed control methods, namely, suicidal germination and also decomposition of stimulant- suggest fascinating views for managing weed pests.

Future Scope: With the advancement of technology, it will be useful to identify and isolate new composites of SLs. Very recently, new compounds like heliolactone, avenaol and methyl zealactonoate had been reported, which may find important application in agriculture. Avenaol and heliolactone are referred to be a non-canonical SLs as they have incomplete ABC-D scaffold. The details of the structure and their stereochemistry will lead to pinpoint the mechanistic details of SL biosynthesis. The SL analogs will play an important role in other biochemical processes other than germination activity. Thus, continuous evaluation of these SL like compounds as factors affecting the branching of AM fungi, regulators of growth and development – will be carried out. Recently, nitro phenlactone was proved to be effective as an agent for seed germinating of *Striga* and *Phelipanche* species.

Research on SLs has developed as a fascinating field that has produced a variety of different signaling models that reflects a complex picture of hormonal perception (Bürger and Chory, 2020). Moreover, it was found that strigolactones exhibit a positive effect on hypodermal passage cells (HPC) density while administration of ABA, ethylene or auxin result in a strong reduction of HPCs and may play a role in shaping exodermal morphology (Liu et al., 2019). SLs and their synthetic analogs have found to be anti-carcinogenic. (Mayzlish-Gati et al., 2015). Hence, it can be stated that strigolactones seem to be a promising compound not only in agriculture, but also in medical sciences.

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Several Variables Affecting to Production of Young Winter Melon (*Benincasa hispida*) Pickle

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ABSTRACT

Winter melon (*Benincasa hispida*) is an important crop with different healthy advantages. It's a rich source of vitamins, dietary fibres, minerals, phytochemical constituents. Purpose of this research focused on a lactic acid fermentation from young winter melon by under different technical parameters such as blanching, salt:sugar ration in immersion, fermentation time to physicochemical, phytochemical and organoleptic properties of pickle. Results showed that young winter melon should be blanched in hot water at 95°C in 8 seconds with 0.45% $CaCl_2$; lactic fermentation in 5.0%:5.0% salt:sugar solution in 13 days to obtain a pleasant taste in good overall acceptance, high total phenolic retention, excellent texture firmness of pickled young winter melon. As a result from pickling, young winter melon will have a longer stability, translucent appearance, firm texture and pickle flavor. Production of pickle from this vegetable can enhance the added value as well as to minimize post-harvest losses.

KEY WORDS: BLANCHING, $CACL_2$, FIRMNESS, LACTIC FERMENTATION, OVERALL ACCEPTANCE, PICKLE, TOTAL PHENOLIC, YOUNG WINTER MELON.

INTRODUCTION

Winter melon (*Benincasa hispida*) belongs to a family of Cucurbitaceae. It's widely consumed in Vietnam for different nutritional and medicinal applications (Nimbal et al., 2011; Zaini et al., 2011; Nguyen et al., 2019). It's highly prefered due to its abundant of vitamins, dietary fibers, antioxidant compounds (Rana and Suttee, 2012; Mandana et al., 2012). It has been utilized to cure gastrointestinal problems, respiratory diseases, heart diseases, diabetes mellitus, urinary diseases as well as other healthy benefits (Rayees, et al., 2013; Rajalakshmi (2018). Vegetable can be safely preserved through lactic fermentation, direct acidification or a combination of these along with other processing conditions (Joshi and Sharma, 2009).

Pickle products by lactic acid fermentation have specific taste with a great healthful advantages. They play an important role in intestinal functions such as modulating immunity, lowering cholesterol and improving lactose intolerance (Isabelle, 2010). Pickling encourages and initiates efficient food processing manipulations and simultaneously minimizes losses due to spoilage and rotten in harvested crops (Sultana et al., 2014). There was not any research mentioned to lactic acid fermentation of young winter melon into pickle. Hence, purpose of this research focused on the effect of various parameters such as blanching, salt:sugar immersion, fermentation duration to physicochemical, phytochemical and overall acceptance of pickled young winter melon.

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MATERIAL AND METHODS

Young winter melons were collected from Vinh Long province, Vietnam. They must be cultivated following VietGAP to ensure food safety. After collecting, they must be conveyed to laboratory as soon as possible for experiments. They were washed under tap water to remove dirty and foreign matters. Besides young winter melon, we also used other materials such as NaCl, $CaCl_2$, sugar, methanol, sodium carbonate, gallic acid. Lab utensils and equipments included knife, weight balance, cooker, fermentator, biuret, micropippetor, centrifugator, spectrophotometer.

Effect of blanching temperature and time to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: Young winter melon were blanched in hot water containing 0.3% $CaCl_2$ in various condition (100/4, 95/8; 90/12, 85/16, 80/20 °C/seconds). The blanched young winter melon would be fermented at ambient temperature in 4.0% salt: 6.0% sugar solution in 7 days. Total phenolic content (mg GAE/100g), firmness (N), overall acceptance of pickled young winter melon would be evaluated to define the optimal blanching formula.

Effect of $CaCl_2$ concentration in blanching to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: Young winter melon were blanched at 95°C in 8 seconds in hot water containing $CaCl_2$ in various condition (0.3, 0.35, 0.40, 0.45, 0.50%). The blanched young winter melon would be fermented at ambient temperature in 4.0% salt: 6.0% sugar solution in 7 days. Total phenolic content (mg GAE/100g), firmness (N), overall acceptance of pickled young winter melon would be evaluated to define the optimal $CaCl_2$ concentration in blanching.

Effect of salt: sugar ratio in fermentation to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: Young winter melon were blanched at 95°C in 8 seconds in hot water containing 0.45% $CaCl_2$. The blanched young winter melon would be fermented at ambient temperature in salt:sugar mixture (4.0%:6.0%; 4.5%:5.5%; 5.0%:5.0%; 5.5%:4.5%; 6.0%:4.0%) in 7 days. Total phenolic content (mg GAE/100g), firmness (N), overall acceptance of pickled young winter melon would be evaluated to define the optimal salt:sugar percentage in fermentation.

Effect of fermentation duration to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: Young winter melon were blanched at 95°C in 8 seconds in hot water containing 0.45% $CaCl_2$. The blanched young winter melon would be fermented at ambient temperature in salt:sugar mixture 5.0%:5.0% in different durations (7, 10, 13, 16, 19 days). Total phenolic content (mg GAE/100g), firmness (N), overall acceptance of pickled young winter melon would be evaluated to define the optimal fermentation duration.

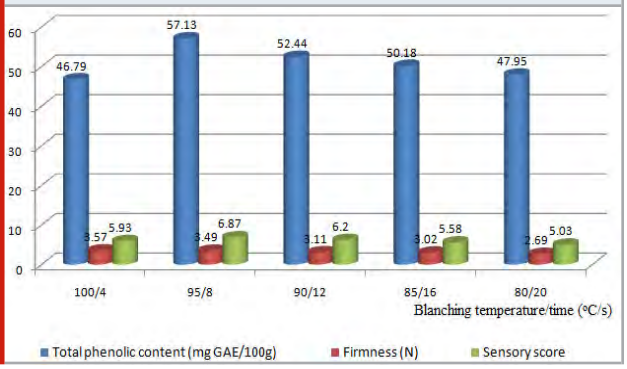
Physico-chemical, overall acceptance and statistical analysis: Total phenolic content (mg GAE/100g) content was estimated using Folin-Ciocalteu reagent procedure. Firmness (N) was estimated by penetrometer. Overall acceptance was evaluated by a group of 11 panelists using 9 point-Hedonic scale. The experiments were run in triplicate with three different lots of samples. The data were presented as mean±standard deviation. Statistical analysis was performed by the Statgraphics Centurion version XVI.

RESULTS AND DISCUSSION

Effect of blanching temperature and time to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: Blanching is normally performed in hot water within a short period of to inactivate enzymes and to eliminate various microorganism present in raw green vegetables (Prakash et al., 2018). Peroxidase is used as an indicator of blanching complement (Badwaik et al., 2015). Hot water blanching significantly delayed tissue lignificaton (Luo et al., 2012). Blanching caused loss of turgor in cells, integrity of the cell membranes and partial degradation of cell wall components. In our research, young winter melon were blanched in hot water containing 0.3% $CaCl_2$ in various condition (100/4, 95/8; 90/12, 85/16, 80/20 oC/seconds).

The blanched young winter melon would be fermented at ambient temperature in 4.0% salt: 6.0% sugar solution in 7 days. Our results were presented in table 1. It's clearly realized that 95°C in 8 seconds was appropriate for blanching of young winter melon. According to Badwaik et al. (2015), the high blanching temperature reduced lightness and long time blanching destroyed firmness of bamboo shoot. Low temperature short time blanching was proven to better product quality in respect of physical characteristics apart from proximate retention.

Figure 1: Effect of blanching temperature and time to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon.

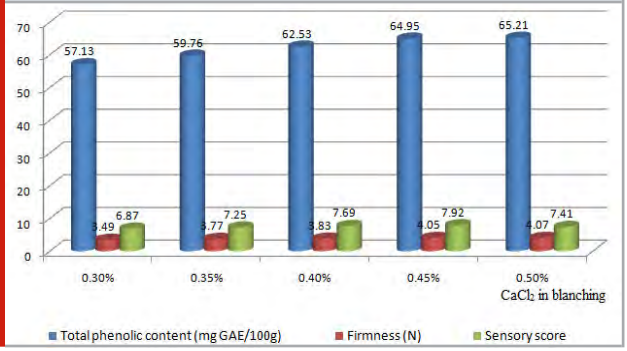


Effect of $CaCl_2$ concentration in blanching to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: High intensity of thermal treatment lead to the significant

loss of phenolic content due to thermal degradation, leaching or diffusion of elements into solution (Goncalves et al., 2010). It could be attributed that during thermal treatment phenylalanine ammonia-lyase and polyphenol oxidase gets inactivated leading to the reduction of phenolic consituents. Texture of blanched vegetable could be attributed to the loss of lignin and cellulosic components of cell wall. There was decrease in lignin and cellulose with increase in time and temperature of blanching (Miao et al., 2011). Softening of tissue after hot water blanching was due to the degradation of pectin with some other biochemical modifications (Badwaik et al., 2015). Blanching altered the chloroplast integrity where the chlorophyll pigments were embedded and results in the formation of pheophytin as the time and temperature of blanching progresses (Llano et al., 2003). Commercial implementation of $CaCl_2$ for fermentation variably resulted in texture and color defects that can impact product quality (Erin and Suzanne, 2018).

$CaCl_2$ played an important role as firming agent at low level to minimize thermal softening and enhance the freshness of blanched vegetable (Martin et al. 2007). $CaCl_2$ included in mild thermal treatment could induce the formation of calcium pectate cross-bonds which stabilized tissue cell membranes (Oms et al. 2010). In our research, young winter melon were blanched at 95°C in 8 seconds in hot water containing $CaCl_2$ in various condition (0.3, 0.35, 0.40, 0.45, 0.50%). The blanched young winter melon would be fermented at ambient temperature in 4.0% salt: 6.0% sugar solution in 7 days. Our results were presented in table 2. The optimal $CaCl_2$ was recorded at 0.45% for blanching of young winter melon. $CaCl_2$ in spinach blanching has been reported to be efficient to E. coli disinfection (Kim et al., 2015).

Figure 2: Effect of $CaCl_2$ concentration in blanching to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon



Effect of salt: sugar ratio in fermentation to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: Salt and sugar provide a natural fermentation for the lactic acid bacteria proliferation and inhibit salt-sensitive spoilage bacteria (Erin and Suzanne, 2018). Salt is one of the most commonly employed agents for food conservation, extending storage stability by reducing water activity (Arghya et al., 2017). Salt is very important as it enhance the preservative, technological and organoleptic properties of food (Brady, 2002). Sugar is metabolized to turn into lactic acid and inhibit the proliferation of pathogens and other non acidic tolerant microorganisms especially aerobic spoilage microorganisms (Nguyen, 2019).

In our present study, different salt:sugar mixture (4.0%:6.0%; 4.5%:5.5%; 5.0%:5.0%; 5.5%:4.5%; 6.0%:4.0%) were examined. Our results were presented in table 3. The optimal salt:sugar was noticed at 5.0%:5.0%. In another report, Susilowati et al. (2018) studied the effect of salt concentration on pH value, total acidity number and microbial characteristic of pickled ginger. They concluded that ginger should be prepared using 2.5% w/w salt with pH value of 3.40, total acidity 0.92% and lactic acid bacteria total counts of 7.56 x 106 CFU/ml.

Figure 3: Effect of salt: sugar ratio in fermentation to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon

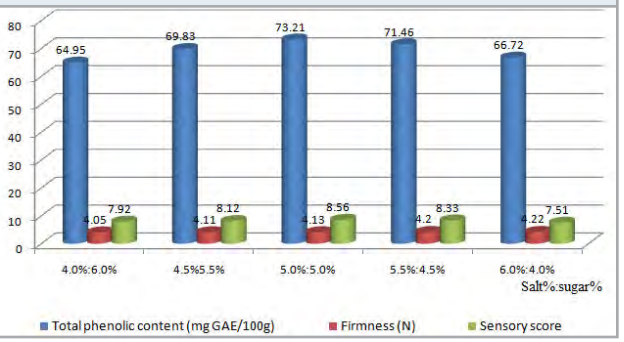
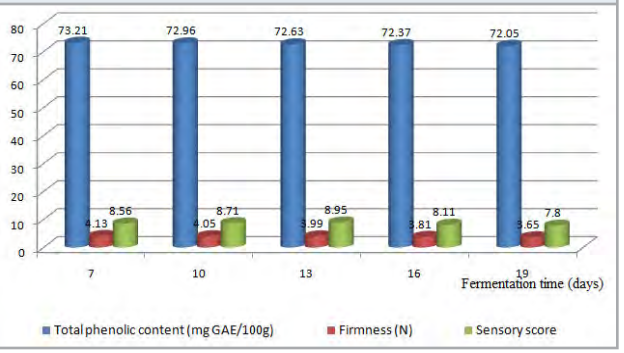


Figure 4: Effect of fermentation duration to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon.



Effect of fermentation duration to total phenolic content (mg GAE/100g), firmness (N) and overall acceptance of pickled young winter melon: The blanched young winter melon would be fermented at ambient temperature in salt:sugar mixture 5.0%:5.0% in different durations (7, 10, 13, 16, 19 days). It's clearly shown that the lactic acid fermentation for young winter melon should be 13 days to obtain the best physicochemical and phytochemical quality as well as overall acceptance. In another report, Susilowati et al. (2018) studied the effect

fermentation time on pH value, total acidity number and microbial characteristic of pickled ginger. They concluded that ginger should be prepared in 5 days fermentation at 26°C.

CONCLUSION

Winter melon is one of the most highly prized vegetables due to its nutritional value and impressive health benefits. Processing and preservation of vegetable into pickle form could reduce price fluctuation of agricultural commodities between the peak harvesting period and off season. High temperature treatment with short duration was most suitable method of blanching to retain total phenolic, textural and organoleptic properties. Salt:sugar ratio, fermentation time were also significantly affected to the quality of winter melon pickle.

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Conflict of Interest: The authors declared that present study was performed in absence of any conflict of interest.

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Chronic and Acute Toxicity of Crude Oils and their Nanoemulsions on the Viability of Larvae of Brine Shrimp, *Artimia salina* Leach

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ABSTRACT

Over the past half century, killing of agricultural pests or insect by synthetic chemical pesticides in the field or post-harvest the crops cause insect resistance and health hazards to man and pollute the environments. There is a great need for developing alternative approaches to control harmful insect pests. *Essential oils* (EOs) and their nanoemulsions (NEs) have broad insecticidal activity against some bests. This study was conducted to investigate toxicological effects of some EOs and their nanoemulsions on the early life stage (24 hr post-hatch nauplii) of brine shrimp *Artemia salina*. Oil of four plants (Neem, *Eucalyptus, Clove* and *Basil*) were collected and their NEs either alone or mixed with Neem were prepared and characterized. The diameter of the nano- particles were 200.1, 211.9, 218,7 and 288.7 nm for Neem, Clove, Basil and Eucalyptus, respectively. All the particles had negative charge. The average diameters were 266.2, 425.1, and 316.1 for Neem+Clove, Neem +Basil and Neem +Eucalyptus, respectively and all the prepared nanoemulsion particles have negative charge. It was found that increasing concentrations of essential oil increased mortality percentage up to 100 %. Moreover, increasing time increased percentage of mortality. The mortality levels were 100 at concentrations of 24, 20 and 16 % after 24, 48 and 72 hr, respectively. The lethal concentration 50 (LC_{50}) of these oils and NEs on *Artemia salina* nauplii was determined after 24 hours of exposure The LD_{50} of the tested neem oil was calculated at 50% mortality level. Similarly, effect of different concentrations of neem oil nanoemulsion on percentage of mortality of *Artemia salina* nauplii after 24, 48 and 72 hr were determined and compared and increasing concentration of neem oil nanoemulsion increased percentage of mortality and increasing time increased also the percentage of mortality. From the previous results, LD_{50} doses for neem oil nanoemulsion were 12, 10 and 8 % after 24, 48 and 72 hr. LD_{50} was calculated after 24 hr for each oil or nanoemulsion prepared from single or mixed oils. LD_{50} values were ranged from 16-46 % for the tested oil and from 12-41% for nanoemulsion of essential oils. Neem oil showed the greatest activities against the tested larva. In conclusion, essential oils of Neem, Eucalyptus, Clove and Basil, applied singly or mixed with neem and their prepared nanoemulsions have broad insecticidal activity and can be used safely against some insect bests.

KEY WORDS: OIL, INSECTICIDAL, NEEM, EUCALYPTUS, CLOVE, BASIL, NANOEMULSIONS, LD50.

ARTICLE INFORMATION

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INTRODUCTION

Synthetic chemical pesticides are mainly used to treat many agricultural pests in the field and post-harvest to protect the crops but with time insect developed a resistance to the synthetic used pesticides. Resistant to Malathion, DDT, deltamethrin and bio-pesticides (Bacillus thuringiensis) was increased which affect human health and need high operational cost. Moreover, these synthetic chemical pesticides cause environmental pollution and many health hazards to warm-blooded animals. There is a need for developing alternative materials to inhibit insect and pests (Isman, 2000 a, b; Yang et al., 2005).Environmental factors may affect the shelf life of nanoemulsion and phyto-nanoemulsions are eco-friendly and effective formulation to combat insects (Sharma et al., 2020).

Therefore, one of the alternative materials with low impact on environment, and safe for human health is natural products or secondary metabolites of plants. More than 2400 plants belonging to 189 families were found in nature (Rao et al., 2005) and contained thousands of essential oils and active compounds like phenolics, terpenoids and alkaloids which may control insects. Some plants natural products can be used as pest control agents instead of the used and unsafe synthetic pesticides. Generally plant essential oils are considered broad-spectrum insectside that are safe for the environment due to high biodegradation rate by soil microbes (Rajendran and Sriranjini, 2008; Devi & Maji, 2011Rodríguez-González et al., 2019).

Lipophilic characters of essential oils made it suitable to be easy used as toxins, feeding deterrents, repellent, oviposition deterrents to broad diversity of insect pests. The aims of this study was to detect biological activities of some essentials oils and their nanoemulsion against Artemis salina as test organism for their potential uses as alternative materials to control insects pests. A wide range of insects were affected by essential oils and they found that essential oils of Pinus brutia, *Laurus nobilis, Cupressus sempervirens, Lavandula stoechas, L. angustifolia, Eucalyptus camaldulensis* and *Thymus vulgaris* were active against many dangerous insects (Kanat and Hakki Alma, 2003). Also, Sampson et al. (2005) reported the insecticidal activities of 23 oil plants as Bifora, Satureja, Coridothymus, Thymbra and Pimpinella using dosage-mortality bioassays and turnip aphids as test organisms.

According to Raina et al. (2007) orange oil (containing ~92% d-limonene) caused 96 % mortality to Coptotermes formosanus within 5 days due to feeding reduction. Upadhyay et al. (2007) tested 14 oils from *Azadirachta indica, Cinnamomum cassia, Cleome gynandra, Cuminum cyminum, Carum copticum,, Cymbopogon narudus, Eugenia aromaticum* and *Nigella sativa* and LC_{50} values were ranged from 0.85-1.25 µl/ml due to inhibition of oviposition and repellent activity. Ayvaz et al. (2010) reported that essential oils of *Origanum onites, Satureja thymbra* and *Myrtus communis* can be used as efficient insecticides. After 24 hr of application, the essential oils of O. onites and S. thymbra at 9 and 25 µl/ml showed inhibitory effects on some insects with 100% mortality level. Also, the same effect was recorded by *Ebadollahi* and Ashouri (2011) for *Achillea millefolium, Artemisia dracunculus* and *Heracleum persicum* with mortality levels of 100% at 50, 65 and 80 µl/ml. The LC_{50} values of some essential oils from A. dracunculus, *A. millefolium* and H. persicum were 22.24, 34.80 and 36.96 µl/ml after 24 h and the LC_{50} values decreased with increasing exposure times. *Essential oils* obtained from Carum carvi and Thymus vulgaris had LD50 of 197 and 250 µg/ cm^2, respectively. The Insecticidal action of *Lavandula hybrida*, *Rosmarinus officinalis* and *Eucalyptus globulus* oils and of their major constituents was reported by Papachristos et al. (2004) and with LC_{50} values was ranged from 0.8 to 47.1 mg/l.

Application of EOs in industrial, agriculture is difficult due to their no solubility in water but formation of nanoemulsions from these oils entranced the solubility. The use of nanotechnology in agriculture and crop protection (Khot et al., 2012; Pavela and Benelli, 2016) is needed to overcome the EOs low water solubility (Donsi et al., 2012). *Encapsulation* of EOs in the form of oil-in-water nanoemulsions (oil droplet <100 nm) could be an effectively possible solution for the mentioned difficulty (Suresh-Kumar et al., 2013).The prepared organic nanoemulsions have drawn a great deal of interest to different sectors with a great number of applications in drugs, pharmaceutical, nutraceuticals, food and agriculture products, and cosmetics formulation. This study aimed to screening of some essential oil or their prepared nanoemulsions for insecticidal activities against using *Artimia salina* as test organism.

MATERIAL AND METHODS

The used essential oils: The essential oils of Neem, Clove, basil and *Eucalyptus* were purchased from local markets, Jeddah.

Preparation of Nanoemulsions: Nanoemulsions from EOs Neem (*Azadirachta indica*) Clove (*Syzygium aromaticum*), Basil (*Ocimum basilicum*) and *Eucalyptus (Eucalyptus globulus)* were prepared. The NEs were prepared using the High Pressure Homogenization (HPH) technique (Nenaah, 2015). The oil samples were diluted with a large amount of water (ratio 1:100) followed by the addition of the surfactant mixtures of Tween 80 and span 20 at a constant ratio of 2:1, respectively. Pre-emulsions were obtained by high speed stirring using an Ultra Turrax T25 (IKA Labortechnik, Jahnke und Kunkel, Germany) at 24,000 rpm for 5 min. Then, the pre-emulsions were passed 5 times through an orifice high pressure homogenizer Nano DeBEE Electric Benchtop Laboratory (BEE International, USA) at 300 MPa. The resulting Nanoemulsions formulas were stored at room temperature, 25°C until used.

Detection of size and morphological characterization of nanoemulsion droplets using Transmission Electron

Microscope (TEM): The particle size analysis and morphology was determined using Transmission Electron Microscope at King Abdulaziz University, Faculty of Science. Three different diameters of each particle were determined and mean value was calculated and standard deviation (SD) was determined (Nenaah, 2015).

Preparation a mixture of 2 oils: By mixing the Neem oil with the other tested oils (*Clove, Basil*, and *Eucalyptus*) at a ratio of 1:1, v/v.

Preparation a mixture of 2 nanoemulsions: Proper fraction of 2 essential oils at ratio 1.1 and distilled water were mixing and heated, followed by the addition of the surfactant mixtures of Tween 80 and span 20 at a constant ratio of 2:1, respectively. By the addition of the warm T80 drop wise along with vortexing. The resulted mixture was heated at high temperature up to 80°C with continuous mixing until one phase emulsion was produced. After that, the warm S20 was added drop wise to the previous mixture with a continuous mixing. This resulted solution was mixed and heated continuously until a clear and transparent solution gets formed (Al-Sowayigh et al., 2019).

Characterization of the prepared NE formulation: Samples were quantified at 25 ± 0.2°C by Zetasizer (3000 HS, Malvern Instruments, Malvern, UK). The oil phase was assumed to have a refractive index of 1.45 and the water phase of 1.33. All the produced formulas were acidic in nature (Al-Sowayigh et al., 2019).

Toxicity test: The cytotoxicity of the purified metabolites were determined using brine shrimp lethality test (Meyer et al., 1982). The tested oil in DMSO at varying concentrations were incubated with the brine shrimp larvae in sea water, and control brine shrimp larvae were incubated in a mixture of sea water and DMSO only. After 24 hr, 48hr or 72 hr the average number of larvae that survived at each concentration was determined and LD50 was calculated (Aly and Gumgumji, 2011).

Statistical analysis: All data were expressed as mean ± standard deviation (±SD). Statistical analysis was performed with two-way analysis of variance (ANOVA) and t-test. The statistical significance difference was considered when p-value≤0.05.

RESULTS AND DISCUSSION

Four tested plant oils named Neem, Clove, Basil and Eucalyptus were collected and their nanoemulsions were prepared and characterized. The average diameter of particles were measured using Zetasizer and compared. The average diameters were 200.1, 211.9, 218,7 and 288.7 nm for Neem, Clove, Basil and Eucalyptus, respectively (Table 1, Figure 1). All the particles had negative charge. Similarly, the mixtures of either Clove, Basil or Eucalyptus with Neem were prepared and their nanoemulsion were formed and characterized. The average diameters were 266.2, 425.1, and 316.1 for Neem+Clove, Neem +Basil and Neem +Eucalyptus, respectively (Table 2) and al the prepared nanoemulsion particles have negative charge. The average diameters of two mixed nanoemulsions were higher compared to control oil at p≤ 0.05.

Figure 1: Particle size and zeta potential distribution of neem oil NE determined by the Zetasizer.

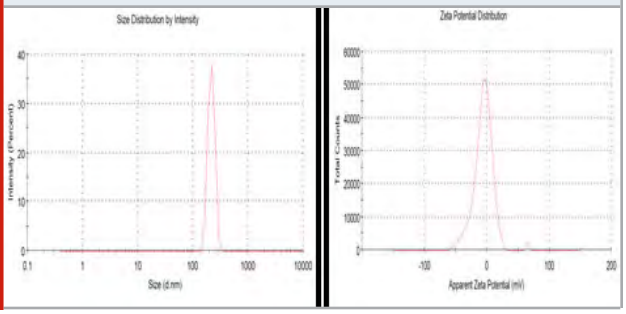


Table 1. The physical characterization of the different NE formulations measured by the Zetasizer.

Formulation	z-average diameter (nm)	CV%= Coefficient of variation	Zeta potential (mV)'
Neem	200.1	11.1	-5.25±1.23
Clove	211.9	7.31	-3.09±1.53
Basil	218.7	13.39	-1.27±1.02
Eucalyptus	288.7	19.39	-1.11±1.09

Table 2. The physical characterization of the mixed NE formulations measured by the Zetasizer.

Formulation	z-average diameter (nm)	CV%= Coefficient of variation	Zeta potential (mV)'
Neem + Clove	266.2	9.29	-3.22 ±1.54
Neem + Basil	425.1	10.40	-1.12±1.24
Neem + Eucalyptus	316.1	41.16	-1.19±1.45

The effect of different concentrations of neem oil on percentage of mortality of *Artemia salina nauplii* after 24, 48 and 72 hr were determined and compared (Figure 2). It was found that increasing concentrations increased mortality percentage up to 100 %. Moreover, increasing time increased percentage of mortality. The mortality levels were 100 at concentrations of 24, 20 and 16 % after 24, 48 and 72 hr, respectively. The LD50 of the tested neem oil was calculated at 50% mortality level (Figure 3). Similarly, effect of different concentrations of neem oil nanoemulsion on percentage of mortality of Artemia salina nauplii after 24, 48 and 72 hr were determined and compared (Figure 4). As shown in Figure

4, it is clear that increasing concentration of neem oil nanoemulsion increased percentage of mortality and increasing time increased also the percentage of mortality. From the previous results, LD_{50} doses for neem oil nanoemulsion were calculated and they were 12, 10 and 8 % after 24, 48 ad 72 hr.

Figure 2: Effect of different concentrations of neem oil on percentage of mortality of *Artemia salina* nauplii after 24, 48 and 72 hr.

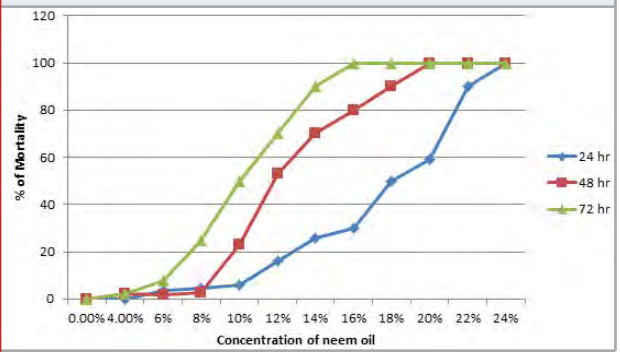
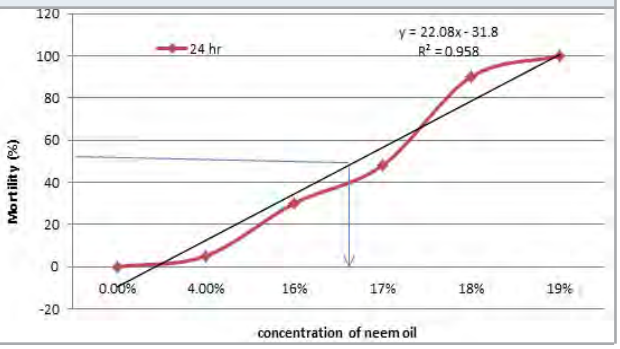


Figure 3: LD_{50} of neem oil detected using Artemia salina after 24 hr of incubation.



For each tested oil or nanoemulsion, different concentrations were prepared and LD_{50} was calculated after 24 hr for each oil or nanoemulsion prepared from single or mixed oils. Concerning the tested oil or their mixture with neem oil, the LD_{50} values were ranged from 16-46 % and the LD_{50} calculated values for nanoemulsion of essential oils were decreased to be ranged from 12-41%. The differences between lethal concentrations of the tested essential oils alone or mixed of two oils and their prepared nanoemulsions was significant at $p \leq 0.05$. Furthermore, there is a significant difference between the activities of the tested oils (LSD 11.08) and their prepared nanoemulsion (LSD 9.9).

There is an increasing request for new active materials for pest control with low adverse effects on human health and the environment. Essential oils played significant roles in plant protection against insect pests and they were simply extracted, biodegradable, ecofriendly, safe for the environment, soil and water with little or no toxicity for man, fishes and birds (Misra and Pavlostathis, 1997; Isman, 2000a, 2006, Isman and Machial, 2006; Bakkali et al., 2008). Essential oils was prepared in dimethyl sulfoxide (DMSO) which is non-toxic emulsifying agent, enhance the penetration of the waxy layer (cuticle) of the insect. Thus, essential oils can act as rapid contact material for insects. The oil of Eucalyptus possesses a wide spectrum of biological activity including anti-microbial, fungicidal, insecticidal/ insect repellent, herbicidal, acaricidal and nematicidal (Batish et al., 2008).

Neem oil is a vegetable oil obtained from the fruits or seeds of the *Azadirachta indica* which is known as neem tree. The evergreen tree of neem is endemic to many areas in the tropical region. The previous oil has many uses in agriculture and medicines. *Ocimum basilicum* (Basil) and *Cymbopogon winterianus (Citronella)* essential oils were used for best control at doses 60 -120 µl/l and Basil and *Citronella oils* exhibited similar patterns of insecticidal activity over the insects (Rodríguez-González et al., 2019).

Figure 4: Effect of different concentration of neem oil Nanoemusions on the percentage of mortality of *Artemia salina* nauplii after 24, 48 and 72 hr.

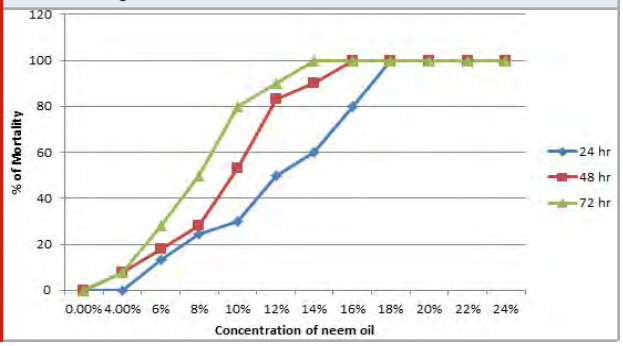


Table 3. The calculated LD50 of the tested oils and their nanoemulsions after 24 hr of incubation.

Tested oil (control)	LD_{50} (%)	Tested oil nanoemulsion (NEs)	LD50 (%)
Neem	16.7±1.8	Neem	12.4±3.1*
Clove	32.33±1.4	Clove	23.9±2.9*
Basil	59.1±4.6	Basil	41.2±1.9*
Eucalyptus	46.2±6.8	Eucalyptus	33.3±7.0*
Neem + clove	14.2±1.1	Neem + clove	10.1±1.9*
Neem + Basil	40.1±3.5	Neem + Basil	30.9±2.9*
Neem + Eucalyptus	40.1±7.9	Neem + Eucalyptus	23.7±5.1*
LSD : 11.0897	LSD: 9.9812		

*: significant results compared to control oil

Eugenol was the major compound of Clove essential oil which act as a promising alternative insecticidal material under storage conditions for control insect pests with 100% mortality level after 48 h with 17.9 -

35 µl/g. The LC50 was calculated to be 9.45 µl/g for *A. obtectus* (Jairoce et al., 2016). Many of the widely used pesticides which are applied in agricultural had low water solubility and highly hydrophobicty, thus, they must be loaded within suitable carrier before use. Several natural products with insecticidal activity have poor water solubility, including triterpenes, and nanotechnology has emerged as a good alternative to solve this main problem Thus, oil-in-water nanoemulsions were used to prepare some pesticides to ensure the efficacy, strong surface adhesion, high penetrability, and many applications. Nano size of droplets amplifies and affect the biological behavior of the nanoemulsions; characteristics of ideal nanoemulsion are low diameter, low viscosity and high zeta potential. Higher stability is dependent on the quantity and composition of surfactants. Environmental factors may affect the shelf life of nanoemulsion and phyto-nanoemulsions are eco-friendly and effective formulation to combat insects (Sharma et al., 2020).

Mentha piperita EO nanoemulsion could be easily prepared through high-energy ultrasonication process and the formulated nanoemulsions were physically stable over 12 months, which makes them interesting candidates for practical applications. EO of M. piperita could be used to kill cotton aphid *A. gossypii* pest and improve food production. The higher concentration of *M. piperita* nanoemulsion caused significantly higher mortality in the adult cotton aphid. Contact toxicity LC50 of the synthesized nanoemulsion in all formulation concentrations varied between 3852 and 3941 ppm, under laboratory conditions (Fernandes et al., 2014).

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Impact of an Educational Intervention in Developing Knowledge, and Awareness of Radiation Therapy Among Families Having Pediatric Oncology Patients

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ABSTRACT

The present study aimed to identify the knowledge, social, physical and psychological effects of children with leukemia and its relationship to gender variables, age, length of treatment, and site of residence. Here comes the role of the psychological counselor to deal with this disease and how to control the situation and the situation in a way that enables the patient to overcome the disease and alleviate the problems that he suffers from. The present study aimed to identify the knowledge, social, physical and psychological effects of children with leukemia and its relationship to gender variables, age, length of treatment, and residence location. The researcher used the questionnaire as a tool to learn the effects. The present study will ad-dress this need by gathering insight into the experience, knowledge and perceptions of fami-lies having children diagnosed with cancer and received the radiation therapy and investigat-ing the impact of an educational intervention in developing their knowledge and answering their quires about the safety of their children undergoing radiation therapy. Also, the article investigates the impact of an educational program in developing parents' knowledge and awareness regarding radiation therapy safety.

It is concluded that there are many obstacles to assessing the needs of a caregiver or patient that may include: lack of clarity about best prac-tices needed to identify needs; Identifying possible levels of performance in every need; Measuring the importance of needs; The correlation of needs assessment with satisfaction and quality of life, and the complex relationships between needs themselves; The extent of the use of needs assessment data when designing care programs; The efforts made to evaluate and follow up on improvement. The assessment directed to uncovering the needs of caregivers for cancer patients should focus on the following three dimensions: the experience of total sabo-tage in life, the extent to which the positive outlook remains, and the attempts made to keep alive. It is recommended that Further research is needed to evaluate outcomes and determine educational approaches that will produce positive changes in nurses' attitudes toward, knowledge of, and application of complementary therapies.

KEY WORDS: ONCOLOGY, RADIATION THERAPY, PEDIATRIC.

ARTICLE INFORMATION

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INTRODUCTION

With the shift to health care and its partial transmission to the home, many cancer patients in various stages of the disease are accompanied by the complications of their needs, they receive care inside the home from (and thus), it is necessary to evaluate the needs of the people with the same family of children with the same family.The medical team and social and psy-chological care professionals know the needs of the caregivers, how important those needs are, how satisfied they are with the satisfaction of those needs they perceive and feel in order to achieve a complete healing process for the patient and to prevent difficulties that he may be exposed to.

The family system and its members. The current study seeks to reveal the needs of caregivers for children with cancer patients and to identify the importance of those needs and the extent to which these needs are met and satisfied and satisfied with that from their point of view. By comparison with adult cancer, pediatric cancer is relatively rare as a disease. Nevertheless, around 100,000 cancer-related deaths in children are reported each year, most of them in de-veloping countries (Al Mutlaq et al., 2015).

In Saudi Arabia, cancer is a significant factor contributing to mortality and morbidity in chil-dren, and, as such, represents an important public health issue. A retrospective study using data from the Saudi Cancer Registry collected between 1999 and 2008 found that 8% of all cancer cases developed in children, and their incidence rose over the time period investigated. The most common tumor types were leukemia (34.1%), lymphoma (15.2%), brain (12.4%) and kidney malignancies (5.3%). Notably, the group of very young children and infants (from birth to age 4) were those with the highest incidence of cancers (Al Mutlaq et al., 2015). By focusing high-energy radiation from X or gamma rays, or released by fast-moving protons, radiation therapy acts to damage cellular DNA, thus destroying the cancerous cells (Kid-shealth, 2016).

The beam also includes a healthy area surrounding the tumor, to reduce the likelihood of re-currence due to migration of cancerous cells into the adjacent tissues. Therefore, normal cells can also be affected by the radiation process, even though they have better repair mechanisms. To offer patients the highest chance of curing or shrinking malignancies while minimizing side effects, a high dose of radiation therapy is aimed at the tumor, with a minimal dose delivered to the surrounding, healthy cells. In children, the healthcare team needs to carefully monitor the doses administered in order to protect the non-malignant tissues. Radiation oncologists may prescribe different types of radiation therapy, depending on the tumor type, size and stage; location in the body; the patient's overall health, medical history and comorbidities; and, not in the least, the patient's age (Lawrence et al., 2008).

In children, radiation therapy is used in a range of tumors, including brain, Wilms tumor, and head and neck cancers (Kid-shealth, 2016). In addition, the management plan is personalized to address the child's disease characteristics and treatment needs; for example, radiation may be offered as standalone treatment, adjuvant to surgery, or in combination with chemotherapy. Radiotherapy, or the combination of radia-tion and chemotherapy, may show increased effectiveness in some cancer types, albeit at the risk of increased side effects (Lawrence et al., 2008).

Most children receive external radiation therapy, which uses high-power equipment focusing on the affected area. In this case, the radiation dose is split over a number of sessions called fractions, allowing the recovery of healthy tissues between fractions. Pediatric patients with cancers of the head and neck, thyroid, uterus or testes may be prescribed internal radiation therapy (brachytherapy), where a radioactive substance is implanted at the site of the tumor, or in some cases swallowed. As with most anti-cancer therapies, radiation therapy is often accompanied by side effects, some of them acute – developing during treatment, and subsid-ing soon thereafter – and others chronic – occurring months or even years after treatment has ended (Lawrence et al., 2008).

Acute side effects include site reactions such as skin irritation, or damage to the exposed tissues (salivary glands, hair loss, and lower abdomen problems). Prescribing a radioprotective medication may help ease these side effects, and clinical trials are underway to test such medications. Among chronic side effects, long-term damage to the targeted area can result in fibrosis, infertility, damage to bowels, and the emergence of a se-cond tumor. In children who have received radiation therapy, the risk of a second cancer is higher than in adult patients; for instance, the risk of developing breast cancer was found ele-vated in girls who had received radiation therapy to the chest for Hodgkin lymphoma (Travis et al., 2008).

As part of the patient-centric approach to cancer management, the radiation oncologist will take into account a child's genetic factors, family history, presence of comorbidities, and any health or lifestyle issues, before prescribing a course of treatment or establishing a dosing regimen, and will review the treatment plan based on careful monitoring of the child's re-sponse to radiation therapy. Treatment personalization is a cornerstone of the patient-centered approach to care, and that is especially relevant to cancer, a multi-faceted disease that has a wide range of objective (external) and subjective (internal/psychological) implications. Among pediatric patients, the caretaking aspect of cancer treatment has even higher priority than in the overall cancer patient population. This underlines the role of the oncology health team and of oncology nurses in particular, not only in delivering the clinical aspects of care, but also in handling the emotional implications of diagnosis and therapy (Skilbeck and Payne, 2003).Communication between patients/families and healthcare professionals has been identi-fied as a key barrier to implementing a patient-centered approach in Saudi hospitals (Aljuaid et al., 2016, Hani Almalki, 2020).

In cases such as those of pediatric cancer, communication needs to be tailored to children's level of understanding and coping mechanisms (Seth, 2010). Simple wording, imagery, and child-friendly materials that convey essential information about the disease, the aims of thera-py, milestones of the treatment journey, and advice for children undergoing treatment, can go a long way in promoting a sense of control and clarity. For children's parents, improving communication relies on full transparency around the diagnosis and available treatment op-tions for those families that require it, and managing expectations regarding treatment re-sponse and side effects (Seth, 2010). In Saudi Arabia, patients' preference for and access to full disclosure of the cancer diagnosis and disease information was demonstrated in a pro-spective survey of 332 Saudi cancer patients from King Fahd University Hospital (Al-Amri, 2010).

The oncology health team should also reach out to children's patients or careers to gain a comprehensive understanding of the patient and formulate a treatment plan that addresses and accommodates their needs (Seth 2010). It has been established that poor communication be-tween healthcare practitioners and patients leads to suboptimal patient outcomes and patient dissatisfaction with the healthcare system (Bonds et al., 2003). However, clinicians and nurses often lack the skills needed to communicate in distressing situations, as they fear their ability to manage the emotions expressed by patients may be limited in such scenarios. As a result, they may choose to prioritize practical care and information above emotional support (Tay et al., 2011).

Other communication issues stem from a paternalistic, medicalized outlook to healthcare, which was proposed to have a cultural component in an Indian study of pediatric cancer pa-tients and families (Seth, 2010). In addition, organizational factors, culture, tradition and ex-pectations, as well as nurses' heavy workloads, can all contribute to a decreased efficiency of communication, as was shown in an Iranian survey (Fakhr-Movahedi et al., 2011, Hani Almalki, 2020).

Patients' and families' religious beliefs also shape the way they cope with the cancer diagno-sis and their perception of self-efficacy in managing the often-strenuous treatment journey that accompanies it. In studies examining the role of religion in helping patients cope with major illnesses including cancer, subjects commonly indicated that religious beliefs and prac-tices were powerful sources of comfort, hope and a sense of purpose (Koenig, 2002). A survey of 39 Muslim breast cancer survivors found that spirituality was women's primary source of psychological support, although all participants were also actively engaged with their medical treatment (Harandy et al., 2010). Finally, the level of family support was shown to increase the likelihood of patients undergoing and adhering to their cancer treatment (Grunfeld et al., 2001; Osborne et al., 2005; Morimoto et al., 2010).

Significance of The Study: Although research has shown that when a child is diagnosed with cancer and treatment starts, the whole family is affected (Flury et al, 2012), Soanes et al, (2009). Parents describe their lived experience of going through the child's cancer treatment as a daily struggle in which the family's normal daily life is disrupted and they have to focus only on the ill child (Björk, Wiebe, and Hallström (2009). It is a taxing period, and the entire family needs support to ease their burdens and get through the crisis (Björk, Wiebe, and Hallström (2009). Olsen, and Harder (2009). Furthermore, in a study done Ångström-Brännström et al., (2015) asking parent about their suggestion to improve the radiation therapy for their children, they concluded that parents need for information from physicians and the staff at the radiotherapy unit.

They want to have repeated information about their child's treatment, as questions arise during treatment and they want to understand what is happening especially when they are seeing the image from the radiation–how large a wound it produces and a little about what zone is irradiated, and so on.. Parents can re-explain information, encourage and support their children as they go through their daily treatment with radiotherapy (Delany, and Conwell (2012). Moreover, the parents' own emotional distress can be relieved by being involved in providing this information to the child and by seeing their child relaxed and calm rather than scared and refusing radiotherapy procedures, (Shrimpton et al., 2012) and, Klosky et al., (2007).

Therefore, the current study will look at investigating the effect of an educational interventions in developing parents' knowledge about radiation therapy since the importance of parental involvement before, during and after radiotherapy is highly recommended. It is aimed at investigating the impact of an educational program in developing parents' knowledge and awareness regarding radiation therapy safety.

Patients with a family history of cancer had an increased level of agreement with their treating physicians on their treatment plans compared with patients without such a history. This increase in the level of agreement could be associated with a patient's previous experience with the shock of a cancer diagnosis of a family member. A patient's ability to absorb the emotional shock of a family member's diagnosis might result in a better acceptance of the diagnosis in oneself. Therefore, collection and documentation of a patient's family history can be used as a tool to estimate a patient's understanding of the treatment plan. Patients with a family history of cancer are more likely to agree with their treating physicians, as suggested by (Hani Almalki, 2020).

Radiotherapy (RT) is the standard of care following breast-conserving operation in breast cancer patients. The neutrophil-to-lymphocyte ratio (NLR) reflects the systemic change caused as a result of the radiotherapy. We aimed to evaluate the association between RT and the change in NLR following the receipt of RT, and to investigate the prognostic impact. We retrospectively

reviewed NLR values of breast cancer patients taken before the administration of the first and the last session of RT. The cut-off point for the NLR was determined using the Youden index and receiver operating characteristic (ROC) curve within the training set. Recurrence-free survival (RFS), distant metastasis free survival, and overall survival were the main outcomes (Chang Ik Yoon, 2020)

1. Research Hypothesis and Questions: The current study was examined the effectiveness of an educational program targeting families having children treated with radiation therapy to support or reject these 4 hypotheses:

1. Radiation safety educational program is effective to increase Parents'/families' understanding and acceptance of their children's cancer diagnosis and care plan, and level of support in implementing this plan, regarding radiation therapy
2. This educational program will improve the families' attitudes toward radiation therapy so, they can teach and predict the sequences and complication that may happen to their children.
3. Radiation safety educational program is not effective to increase Parents'/families' understanding and acceptance of their children's cancer diagnosis and care plan, and level of support in implementing this plan, in particular with regard to radiation therapy.
4. There is no association between families' knowledge and attitudes toward Radiation therapy treatment and their sociodemographic characteristics.

2. Methodology

Study design: A Quasi-experimental, specifically "one group pre-post-test" design will be used in the current study to achieve its objectives.

Setting: study assessing the experience, knowledge and attitudes of having families'(parents) of pediatric oncology patients on key aspects of their cancer diagnosis and radiation treatment at three hospitals in Jeddah, Saudi Arabia.

Subjects: the study was enrolled 65 participants by using convenient sampling technique and they are literate.

3. Validity and Reliability: The instrument was translated into Arabic and back translated into English, verifying whether the translation covers all aspects of the original English version of the questionnaire or not. To ensure the face validity of the final translated Arabic version of the questionnaire it was evaluated by experts who were selected based on their qualifications and experience in nursing research and education. Then, the tool was piloted and tested by 10 participants to identify ambiguities, the time required and any difficulties that might be encountered by the participants in reading or understanding. The reliability of the questionnaires was calculated and Cronbach Alpha for knowledge and attitudes questionnaires were reported later.

Data Management: The data were analyzed using the most resent version of SPSS. Data were reported using descriptive statistics in the form of frequencies, percentages, means and standard deviations. A paired t-test was used to analyze the total scores of the participants' responses on the pre-test and the post-test. Contributors' sociodemographic and knowledge differences were analyzed using Chi Square test ($\chi 2$). Pearson R was used to test the correlation between families' knowledge and sociodemographic variables. The significance level is pre-set at $p<0.05$.

Program description and data collection procedure: When a child has been diagnosed with cancer and has to undergo radiotherapy, parents a devastating feeling of shock and chaos and have to come to terms with the fact of a life-threatening illness. Parents can experience overwhelming feelings like feeling sick and mentally exhausted, as well as uncertainty, distress and fear of the illness and the radiotherapy treatment: They feel that everything concerning the disease and treatment is happening so fast that they cannot absorb the information or understand what is happening. The importance of parental involvement before, during and after radiotherapy must not be underestimated. Parents can re-explain information, encourage and support their children as they go through their daily treatment with radiotherapy. The parents own emotional distress can be relieved by being involved in providing this information to the child and by seeing their child relaxed and calm rather than scared and refusing radiotherapy procedures.

The objectives of the educational program were:

1. Develop parent' knowledge about meaning of radiation therapy
2. Assist parents to recognize different types of radiation therapy (internal and external treatment)
3. Develop parents' awareness by the short term and long-term side effect and complication of radiation therapy.
4. Teach parents' the appropriate methods which can help them to teach and protect their kids from emotional exhaustion and how their children feel better during treatment as well when to call for medical help.
5. Empower parents with the skills of communication, listening, and how to develop a strong relationship with their children to break silence of their fear and motivate them to talk about their fears, emotional pain and traumatic experiences.

Data Collection procedure: Pre-test administration:

1. A letter with all details of the educational program was explained for parents during their presence with their children to motivate them to participate in the study
2. Ethical issues were raised by taking verbal and written consent for participation from every family after explaining the aim of the study and confirming confidentiality of their data.
3. Participants were asked to fill the questionnaire at

the beginning of the first day of data collection (i.e., before the theoretical session starts).

Program description: The Data were gathered after receiving the approval. The participants were requested to fill up the questionnaires pre, and post the educational program. The educational training program consisted of 2 days (6 consecutive sessions), each part involves 3 sessions and each session will be 45 minutes to 60 minutes.

First part: Theoretical part about radiation therapy including definition, types of radiation therapy and difference between internal and external radiation treatment, how radiation is given and what happens during external and internal radiation therapy. Moreover, the parent was informed regarding the common and long-term side effects of radiation. Moreover, the importance of caring behavior for your child and how you can convey.

The second part: This part was demonstrated for the different ways of successful communications with our children to build a trust relationship that motivate them to accept learning by parents and they can inform regarding any emotions without fear. In addition to, the effective methods for parents to protect their children emotional exhaustion and depression.

a. Encouraging children to talk about their feeling and ask questions by using active listening b) Provide knowledge to their children and convey acceptance and caring behavior c)Teaching their children, by using simulation games, pictures or may be allowed to enter the room of treatment prior the session with help of medical and nursing staff.

Methods of instructions: Power point presentation, Simulation activities, Photos and audio-visual materials and videos and movies. As regard to the effectiveness of instructional methods that were used throughout the program, many studies suggested that audio-visual materials, video showing, role play and modeling are effective methods in teaching.

Post-test administration: Questionnaires were fulfilled again by the parents after implementing the educational program.

RESULTS AND DISCUSSION

Parents' Background information: 65 parents attend the program. All parents were Saudi and all pediatric patients were Saudis. Table 1 shows the age group for the participating parents which is high between age 40-49 and decrease after that.

Table 2 shows the parents educational level which is high at secondary education level. Also, as shown in Table 3 most of the participant were from Jeddah city.

Table 1. Age group for the participating parents

Age of the parents	%
20-29	22%
30-39	29%
40-49	38%
50-59	11%
Total	100

Table 2. Parents educational level

Not educated	9%
Primary	13%
Secondary	41%
Batcheler	35%
Post graduate	2%
Total	100

Table 3. Parents' Living in Jeddah/or outside Jeddah

City	%
In Jeddah	64 %
Outside Jeddah	36%
Total	100%

Table 4. Parents Information about their child disease at time of discovering the dieses and initial diagnosis.

Parents Information about the disease at time of discovering the dieses and initial diagnosis	%
The initial reaction was shocking at first time hearing the diagnosis	97 %
Awareness regarding the child diagnosis at first time hearing the diagnosis	28 %
Awareness regarding symptoms progression at first time hearing the diagnosis	24 %
Awareness regarding particular tests for cancer at first time hearing the diagnosis	21%
Feeling that there was sufficient information about the dieses available at the time of your diagnosis	29 %
Feeling that there was sufficient information about the treatment plan at the time of your diagnosis	26%
Received psychological counselling before radiation therapy	20%
Feeling that there was emotional support provided by the health team at the beginning of radiation therapy	27 %
Feeling that their child finds have helpful support in coping with radiation therapy	31 %

Table 4 shows Parents Information about their child disease at time of discovering the dieses and initial diagnosis and the high level of shocking at first time hearing the diagnosis (97 %) and it shows the low percentages regarding their information about the dieses, the treatment plans, and sever lack of emotional support at the time of the diagnosis which reflects in their child coping with radiation therapy.

Figure 1: Parents Information about their child disease at time of discovering the dieses and initial diagnosis.

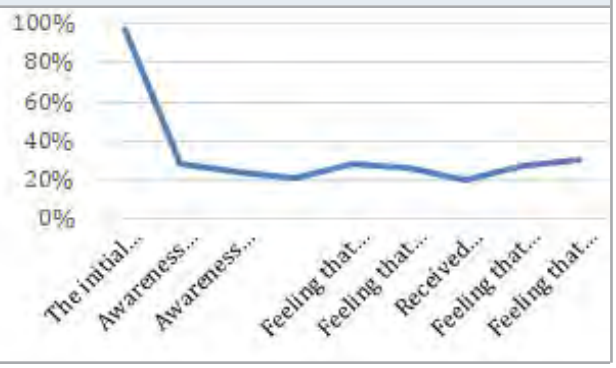


Table 5 compares between the parents Information about the diagnosis and treatment plan before and after the program and it shows improvements in parents information related to understanding regarding their child's disease, treatment plan and coping with their child diagnosis.

Table 5. Parents Information about the diagnosis and treatment plan before and after the program.

Parents Information about the diagnosis and treatment plan	before the program	after the program
Parents understanding regarding their child's disease	34 %	52%
Parents understanding regarding their child's treatment plan	42%	59%
Parents coping with their child diagnosis	31%	43%

Figure 2: Parents Information about the diagnosis and treatment plan before and after the program.

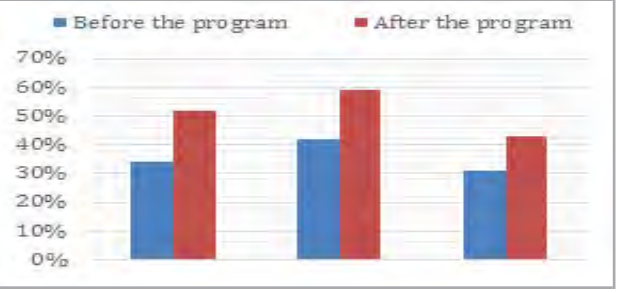


Table 6 shows the importance of parents Information about their child Experiences with radiation therapy side effects and Importance information about their child's overall health. While Table 7 shows the importance of parents Information about their child's common radiation therapy side effects.

Table 6. Parents Information about their child Experiences with radiation therapy.

Parents Information about their child Experiences with radiation therapy	%
parents Information about their child experience any side effects of radiation therapy	96 %
parents Information about their child's overall health	89%

Table 7. Parents Information about their child's common radiation therapy side effects.

Parents Information	%
skin reactions (irritation, redness, sensitivity)	94%
Tiredness	96 %
Hair loss	98%
Appetite status	93%
Sore mouth or tooth decay	92 %
Nausea and vomiting	93 %
Pain	92 %
Sleep disturbance	87%
Infections/fevers recurrence	91%
Mood changes	84 %

Figure 3: Parents Information about their child's common radiation therapy side effects.

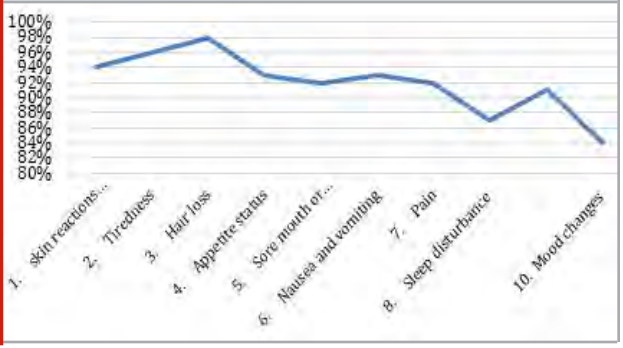


Table 8 compares between the Parents' knowledge about their child Experiences with radiation therapy side effects before and after the program and it shows improvements in level of knowledge and comfortable to ask the medical team regarding their child health status.

Table 8. Parents' knowledge about their child Experiences with radiation therapy side effects before and after the program.

Parents' knowledge about their child Experiences with radiation therapy	before the program	after the program
knowledge toward information provided by (booklet, link to website, simulation session, video)	38 %	63 %
knowledge providing about lifestyle advice to help manage the side effects of radiation therapy	35%	51%
Parents knowledge if their child finds these advices were easy to understand	31 %	46%
Parents knowledge if their child finds these advices were easy to put into practice	32%	53%
Parents knowledge if their child experiences any learning difficulties or memory loss after radiation therapy	43%	55%
Parents feels comfortable asking for advice by the medical team	48%	59%
Parents knowledge regarding the importance of regular monitoring after stopping radiation therapy	45%	61%
Parents feels comfortable to discuss with medical team about treatments available after radiation therapy	47%	64 %

Figure 4: Parents' knowledge about their child Experiences with radiation therapy side effects before and after the program.

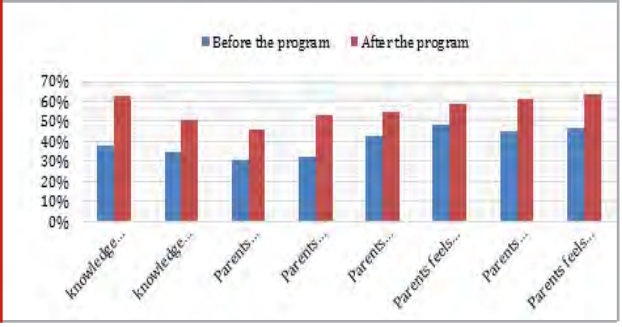
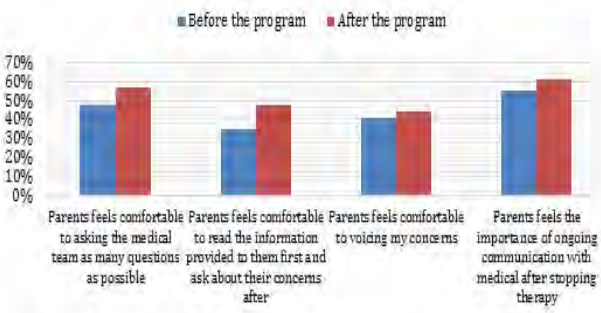


Table 9 compares between the Parents' Parents Information about the communication with the medical team which was improved after the program.

Table 9. Parents comfortable about the communication with the medical team before and after the program.

Parents Information about the communication with with the medical team	before the program	after the program
Parents feels comfortable to asking the medical team as many questions as possible	48%	57%
Parents feels comfortable to read the information provided to them first and ask about their concerns after	35%	48%
Parents feels comfortable to voicing my concerns	41%	44%
Parents feels the importance of ongoing communication with medical after stopping therapy	55%	61%

Figure 5: Parents comfortable about the communication with the medical team before and after the program.



CONCLUSION

The article examined the effectiveness of an educational program targeting families having children treated with radiation therapy to support or reject these hypotheses:Radiation safety educational program is effective to increase Parents'/families' understanding and acceptance of their children's cancer diagnosis and care plan, and level of support in implementing this plan, in particular with regard to radiation therapy, This educational program improve the families' attitudes toward radiation therapy so, they can teach and predict the sequences and complication that may happen to their children., Radiation safety educational program is not effective to increase Parents'/families' understanding and acceptance of their children's cancer diagnosis and care plan, and level of support in implementing this plan, in particular with regard to radiation therapy. There is no association between families' knowledge and attitudes toward Radiation therapy treatment and their sociodemographic characteristics. Finally, the current study looks at investigating the effect of an educational interventions in developing parents' knowledge about

radiation therapy since the importance of parental involvement before, during and after radiotherapy is highly recommended.

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Describing Evidenced-Based Practice and Research Utilization Amongst Critical Care Nurses at a Military Hospital in Jeddah, Saudi Arabia

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ABSTRACT

Evidence-based practice (EBP) is recognized internationally as a foundational element in health care. When delivered in a context of caring and a supportive organizational culture, the highest quality of care and best patient outcomes can be achieved. The study aimed to describe the evidenced-based practice and research utilization amongst critical care nurses at the National Guard Hospital in Jeddah, Saudi Arabia. This study followed a cross-sectional descriptive quantitative approach. The study was conducted in the critical care units of this hospital. in Saudi Arabia. A convenience sampling method including 96 critical care nurses was used. Data was collected using a tool that has established reliability and validity. Descriptive statistics included (mean, SD, frequencies, and percentages) and Inferential statistics were carried out such as (independent t-test, and Chi-square) using the Statistical Package for the Social Sciences.

The biggest barrier was reported as insufficient time on the job to implement new ideas, M= 3.10, from the construct of setting. The lowest barrier was reported as ''I fell the benefits of changing practice will be minimal" M= 2.45. More females (79%) reported the construct of setting as the barrier, M= 2.88. There was a significant difference noted in the four constructs and age, P= 0.00 was noted. For EBP and research utilization to become the golden standard of health care, health care organizations and systems should advocate its use. Even though EBP has been highlighted as a core competency for health care professionals, the uptake of EBP into practice remains a challenge

KEY WORDS: EVIDENCED-BASED PRACTICE, RESEARCH, UTILISATION, CRITICAL CARE NURSES.

ARTICLE INFORMATION

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INTRODUCTION

Evidence-based practice (EBP) is recognized internationally as a foundational element in health care (Lehane et al 2019). EBP has been defined as a problem-solving approach to the delivery of health care that integrates the best evidence from studies and patient care data with clinician expertise, patient preferences and values. When delivered in a context of caring and a supportive organisational culture, the highest quality of care and best patient outcomes can be achieved (Melnyk et al, 2009). The emergence of EBP has been fast spreading within health care and has resulted in changing the way health care is undertaken (All Answer Ltd, 2018).

Determinants of successful implementation of EBP in clinical practice have been described in multiple studies and relate to the individual, the clinical question, the evidence, and the environment or context in which these occur. The successful implementation of EBP is dependent on the individual, the clinical question, the evidence, and the context (Rios & Thabane, 2010; Krupski, et al., 2008;). Several process models that have been developed to guide nurses through EBP. Most are process models that focus on guiding the nurse through the necessary steps however these models have not been consistently and uniformly used (Titler, 2018).

Nurses are the largest health professional groups that can help achieve implementing EBP as they are in direct contact with patients where assessment and care interventions are made daily (Hagdu, Almaz & Tsheay, 2015). Evidence-based nursing practice allows nurses to provide the highest quality care based on the best evidence that can exist, which in turn results in a positive outcome in nursing interventions. To improve current and future patient outcomes it is essential to incorporate an EBP approach in clinical nursing (Elarab, El Salem, Behalik, et al, 2012). Today clinical decision making is driven by EBP which is a theory- derived promoting optimal patient outcomes by incorporating the best evidence, the nurses' experience, and patient preferences (Penz,2006).

However, despite nurses being in a pivotal position to achieve optimal patient outcomes, research findings are often conveyed to researchers and not nurses in the clinical setting. As a result of this, the concept of research utilization was introduced into nursing only in the 1970s (Stetler, 2001). In addition to this, Patelarou, Kiriakouis, Stamou, et al (2017) highlighted that health professionals' adoption of EBP in practice remains limited even though they "are familiar with EBP and believe in its value". Further to this, the healthcare professional has a positive attitude towards EBP however their understanding and skills related to EBP are inadequate. Despite the benefits of EBP, there are many personal and organizational barriers impeding EBP implementation. These barriers can be summarized in terms of the attitude of nurses to undertake research (lack of research knowledge; lack of communication; lack of communication from knowledgeable colleagues; weakness of evaluating the research), organizational constraints (time; lack of authority; unsupportive personnel) and research communication (not readily available reports, research jargon, and literature) (Panagiari, 2008 Shayan et al 2019).

Although there are increased demands for the utilization of research in nursing practice, there are differences in the nurses' education level regarding research utilization (Elarab et al., 2012). Evidence-based practice requires making professional decisions based on systematically gathered evidence drawn from research and experience based on the patients' desires and needs in a specific situation (Dalheim et al., 2012). As nurses are engaging more in EBP assistance is needed in providing greater evidence based guidance to deliver effective care defined by the best research. This will also assist in resolving problems in the clinical setting to achieve excellence in delivering patient care (Al Touby , 2017).

Increased awareness that nursing should become an evidence-based profession has recently become more and more important in several countries around the world (Biesta, 2007). EBP may be more successfully implemented if the interventions overcome identified barriers (Dalheim et al., 2012; Hadid & Barnawi, 2012). As nurses gain EBP knowledge and skills, they realize it is not only practical within the context of their practice setting, but they also develop a passion for their roles as EBP practitioners resulting in them delivering a higher quality of care with improved patient outcomes (Fineout-Overholt, Melnyk, Stillwell and Williamson, 2010).

The Critical Care Unit (CCU) is a high tech, fast-paced environment. Admissions to a CCU is mostly unplanned and the patients are in a critical condition (Hashim and Hussin, 2012). Admissions are due to illnesses which are often life-threatening resulting from trauma, surgery, sepsis or shock where patients are susceptible to dysfunction of multiple organ systems including respiratory, cardiovascular and digestive system (Wright-Myrie, Kahwa, and Dover-Roberts, 2013). The intensive care environment imposes physical, emotional, and cognitive stresses on Critical Care Nurses (CCNs) and they must be adequately trained to deal with rapidly changing technology, which can be both a support and a burden to staff members (Almerud, Alapack, Fridlund and Ekebergh, 2008). CCNs must assess and monitor the patients' physiological responses to treatment, paying close attention to conditions requiring immediate interventions. Patients admitted to CCUs tend to be physiologically unstable, requiring constant cardiac and respiratory monitoring and continuous adjustments of treatment. These challenges require CCNs to be skilled at interpreting, integrating, and responding to a variety of information (Losa Iglesias, Vallejo, and Fuentes, 2010).

There are many reasons put forward to why it is so difficult to provide evidence-based care. "One of the most obvious is the fact that new evidence is being

generated at an ever-increasing rate. It is estimated that nearly one million new articles are posted on PubMed annually" (Loannidis et al, 2018, p: 795). Therefore, health care professionals are faced with the challenge of finding, appraising, and integrating new evidence into the routine practice (Shayan et al, 2019). Further to this, most healthcare professionals are unaware of the poor quality of evidence that results in improper care and wastage of health resources, Therefore, efforts should focus on training healthcare professionals to be more sensitive to the limitations of the evidence, doing critical appraisals and improving communication skills. This will equip health care professionals to effectively summarize and discuss medical evidence with patients to improve decision-making. (Loannidi et al, 2018).

EBP remains limited, although most health care professionals are familiar with EBP and believe in its value (Patelarou et al, 2017). Evidence-based practice and research utilization is a phenomenon that is relatively under-researched within the context of Saudi Arabia (Alqahtani et al, 2018; Alshehri et al, 2017; Gulman et al, 2017; Bahammam & Linkawi 2014). and more specifically within the context of nursing and critical care nursing. Critical care is an area of specialization where patients are physiologically compromised within a highly technical environment. Hence critical care nurses should have a strong knowledge base on EBP guidelines to provide the best possible care. This study was conducted within critical care nursing in the context of Saudi Arabia and has contributed to research within EBP within these contexts.

MATERIAL AND METHODS

The study was conducted at the National Guard hospital in critical care units, Jeddah, Saudi Arabia. This is a tertiary hospital that has 596 beds. Two hundred and one beds are allocated to the critical care units within 10 critical care units. This study followed a cross-sectional descriptive design. The ethical approval of the study (approval NO. RJ15/013/J) was received from the Research Office at King Abdullah International Medical Research Centre. Before the data collection, the informed consent form was signed by each participant of the study. Sampling was completed using a convenience sampling method. The total population of CCNs was 277, however only 96 critical care nurses working in 10 adult critical care units were included in the study. Only critical care nurses with a minimum of six months of experience were included in the study. Data collection was done using a questionnaire that consisted of four sections. Section A represented the demographic section of respondents, section B represented the meaning of EBP, Section C represented barriers to EBP, and research utilization, Section D, represented the participation in research activities.

Section C of the tool was adopted by Funk, Champagne, Tornquist, and Wiese (1991). The original questionnaire consists of 28 items into four subscales namely adopter, organization, research, and communication. The responses were provided on a Likert scale, that was rated from not at all familiar to very familiar (1, not all familiar; 2, to little extent familiar; 3, to moderate extent familiar; 4, to a greater extend familiar; 5, completely familiar). The 28 items on the tool were adopted from the original tool and amended for the Saudi context. This section of the tool was divided into four constructs namely the setting (11 items), research (4 items), nurse (8 items), and presentation (5 items).

Section B, and D were developed by the researchers after a literature review. A pilot test was completed using the entire tool and revealed a Cronbach alpha of 0.90. Those involved in the pilot test were excluded from the final data collection of the study. The pilot test included five critical care lecturers. Section D of the tool has existing reliability of Cronbach alpha of 0.65-0.80 and 0.61-0.71 (Funk, Champagne, Wiese & Tornquist, 1991) and 0.74-0.87 (Kang, 2015). However, the tool was piloted and showed a Cronbach alpha of 0.937. SPSS version 20 was used for data analysis

RESULTS AND DISCUSSION

A total of 96 CCNs participated in the study. 82, 3% of respondents were female. The majority (33.3%) of respondents were between 35-45 years of age. The most common educational qualification among respondents was a bachelor's degree in nursing (69.8%). Most respondents 30.2% had nursing experience within 1-5 years with only 10.4% of respondents having more than 20 years of experience in critical care.

Figure 1: Meaning of EBP

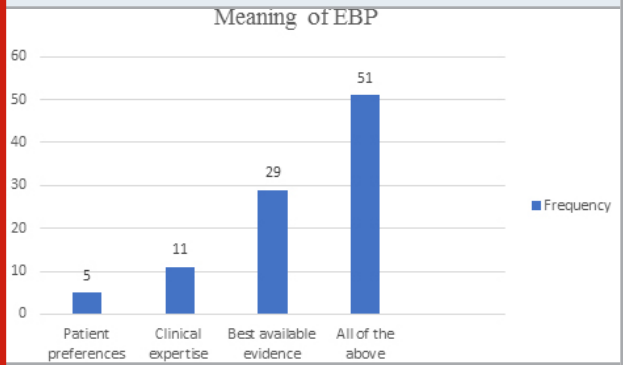

Table 1. Barriers to research utilization and EBP in terms of constructs

Construct	N	Minimum	Maximum	Mean	Std. Deviation
Setting	96	12.00	60.00	34.38	9.07
Presentation	96	6.00	30.00	16.57	4.44
Nurse	96	6.00	30.00	16.31	4.27
Research	96	4.00	20.00	10.84	3.15

Participants were asked about the meaning of EBP and the majority of participants (53%) indicated that EBP includes best clinical expertise, best available evidence, and patient preferences, which is the correct response as EBP includes patient preferences, clinical expertise, and best available evidence.

The above table highlights that barriers relating to the construct of the setting were the biggest barrier, M=34.38 with the construct of research being the lowest barrier, M= 10.84.

Table 3 highlights that even though there is no statistical difference between gender and the barriers to research utilization and EBP, from the construct of setting, females perceived the setting to more of barrier M= 2.88 than males, M= 2.79.

Table 4, highlights that there are significant differences in terms of age between the barriers related to the various constructs. Younger nurses within the age group of fewer than 25 years (4.2%) found the construct of presentation of t research to be the biggest barrier, M= 34.92, with the majority of nurses between 25-35 years (49%), found that barriers related to the construct of research were the biggest barrier, M=34.98. Nurses between the ages of 35-45 years (33.3%), reported that barriers related to the construct of the nurse were the biggest barrier, M=34.97. Finally, the older nurses, between the age group of 45- 60 years (13.7%) reported that the construct of the setting was the biggest barrier, M= 34.82. Hence, within this study, it can be assumed that the younger nurses reported barriers related to research-related issues, whilst the older nurses reported barriers to shortcomings in themselves as nurses and the organization.

Table 2. Barriers related to research utilization and EBP

		Construct	Mean	Std. Deviation
1.	I feel the benefits of changing practice will be minimal	Nurse	2.45	1.239
2.	I am unwilling to change/try new ideas	Nurse	2.53	1.151
3.	The research has methodological inadequacies	Research	2.55	.961
4.	I do not see the value of research for practice	Nurse	2.60	1.235
5.	I am isolated from knowledgeable colleagues with whom to discuss the research	Nurse	2.68	1.110
6.	The administration does not support EBP implementation	Setting	2.68	1.138
7.	I am unaware of the research-	Nurse	2.70	1.087
8.	Research reports/articles are not readily available	Presentation	2.72	.948
9.	I see little benefit for myself	Nurse	2.73	1.128
10.	Access to research evidence is poor (slow or no computers, or databases)	Setting	2.74	1.126
11.	Statistical analyses are not understandable	Presentation	2.75	1.016
12.	The research is not relevant to practice	Presentation	2.76	1.054
13.	The research has not been replicated	Research	2.76	1.074
14.	The amount of research information is overwhelming	Research	2.76	1.023
15.	I am uncertain whether to believe the results of the research	Research	2.77	.968
16.	Implications for practice are not made clear	Presentation	2.80	.902
17.	The facilities are inadequate for implementation	Setting	2.81	.921
18.	The relevant literature is not compiled in one place	Presentation	2.82	1.086
19.	I do not feel capable of evaluating the quality of the research	Nurse	2.83	1.063
20.	There is resistance to make changes in the work setting	Setting	2.83	1.185
21.	I feel the benefits of changing practice will be minimal	Nurse	2.84	.944
22.	Other staff are not supportive of the implementation	Setting	2.84	1.079
23.	The administration will not allow implementation	Setting	2.85	1.161
24.	There is not support or incentives for clinical practice development	Setting	2.91	1.152
25.	I do not have time to read the research	Setting	2.99	.968
26.	I do not feel I have enough authority to change patient care procedures	Setting	3.08	1.262
27.	I feel results are not generalizable to own setting	Setting	3.09	1.077
28.	There is insufficient time on the job to implement new ideas	Setting	3.10	1.147

Table 2 above highlights that only half (50%) of the respondents' participation in research during their time of employment at the present hospital. Only N=33 (34.4%) of respondents participated in research once; while N=10(10.4%) participated in research 2-3 times and N= 3(3.1%) participated in research more than 3 times.

Table 3. Gender differences in terms of the different constructs

Construct	Gender	N	Mean	Deviation Std.	Std. Error Mean
Presentation	Female	79	2.7679	.77782	.08751
	Male	17	2.7353	.55920	.13563
Research	Female	79	2.7089	.82278	.09257
	Male	17	2.7206	.63049	.15292
Nurse	Female	79	2.7236	.74530	.08385
	Male	17	2.6961	.55664	.13500
Setting	Female	79	2.8808	.79726	.08970
	Male	17	2.7941	.53774	.13042

Further to this, it was noted that respondents N= 24 (51.1%) between the age groups of 25-35 years were more active in research participation than all other groups. In addition it was found that more females n= 35 (36.4 %) participated in research than males N= 11 (10.4%). Most respondents N=23(69.7%) who participated in the research were mostly from the adult general CCU whilst only N= 10 (30.3%) respondents from the pediatric CCU took part. In addition, N= 18 (18.7%) respondents searched for information, research or evidence to support their nursing practice several times a week, whilst N= 28 (29.2) searched weekly, N= 33(34.4%) 1-2 times per month, N=13 (13.5%) less than once a month, whilst, N= 4 (4.2%) never searched for any information. Also, only N= 19 (19.8%) of respondents had a subscription to a health journal.

This study highlighted that only 50 % of nurses were involved in the research. In addition, even though 50% were not involved in research, 53% of respondents had the correct conceptualization of what EBP was. The correct meaning of EBP included the best clinical expertise, best available evidence, and patient preferences. Sackett et al, (1996) suggest the best available evidence is, therefore, integration of three factors; clinical expertise, results of high-level systematic, clinical research, and patient preference. However, Hoffmann et al (2013) expanded the definition to include available resources. Further to this, the current study did not highlight any significant correlation between the lack of EBP and research utilization with a lack of understanding of what EBP meant.

Table 4. Barriers related to age

	Paired Samples Test				
		Paired Differences	t	df	Sig. (2-tailed)
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	Age - presentation	34.92194	39.182	95	.000
Pair 2	Age - research	34.98890	38.879	95	.000
Pair 3	Age - nurse	34.97111	39.099	95	.000
Pair 4	Age - setting	34.82539	38.904	95	.000

However, Al-Baghlie and Al-Almaie (2004), found that physicians who had no understanding of what evidenced-based medicine was, were the ones who had a negative attitude towards evidenced based medicine. It is interesting to note that Al- Baghlie and Al-Almaie (2004) reported that"...the poor understanding of evidenced-based medicine could lead to misunderstandings where physicians who misunderstood evidenced-based medicine would be protective of their current way of practicing medicine. Contrary to the findings Mallion and Brooke (2016), reported that even though almost one-third of respondents never heard about EBP which was higher in previous studies, the median total score for EBP beliefs was positive.

Table 5. Participation in research during your employment at the current hospital

	Frequency	Percent
Not at all	50	52.1
Once	33	34.4
2-3 times	10	10.4
More than 3 times	3	3.1
Total	96	100.0

The least reported barrier to research utilization and EBP was " I feel the benefits for changing practice will be minimal" There is a possibility that nurses within this study found that EBP is beneficial. This can be further attributed to the fact that changing practice can be related to the benefits of changing behavior which

can be related to the best possible care for patients. as Greenhalgh et al., (2014) implore, EBP should always have "the care of individual patients as its top priority.

" Further to this, one of the major findings of this study highlighted that the top barrier of research utilization was insufficient time: to implement new ideas and the time to read research More recently, Mallion & Brooke (2016), and Gomes Perieria, da Silva Peixoto de Oliveira Cardoso & Correia dos Santos Cardosa Martins (2012) also reported that the most significant barrier to the implementation of EBP was a lack of time. This is reiterated by Shayan et al (2019) who also highlighted that time is a barrier to the implantation of EBP. The lack of time to conduct research, to read research findings, and to implement new ideas were all barriers of EBP related to time.

Further to this, a lack of time has been related to increased workload. Health care institutions tend to have a culture of busyness that is rewarded and valued, however, it does not encourage nurses to spend time sitting and reading (Dalheim, Harthug, Nilsen, et al 2012; Jordan, Bowers & Morton, 2016). The lack of time to read and document research is well cited in the literature (Brown, Kim, Stichler & Fields, 2010; Breimaier, Halfens, & Lohrmann, 2011). According to Barends et al (2017), time is important for EBP to be realized as generating research evidenced and the use of evidence is time-consuming. Besides, numerous studies highlight that an increased workload reduces time on the job for EBP related activities (Khammarnia et al, 2015; Adib-Hajbaghery, 2007 Ebrahimi et al, 2017).

The findings of this study highlight the top five barriers, as well as other barriers to research utilization and EBP, was related to the construct of the setting. Most respondents reported that setting or the organization related factors were the biggest challenges. These barriers included "other staff members not being supportive of implementation", "administration will not allow implementation" and "there is no support or incentives for clinical practice". Kaplan et al., (2014) reported that support from administrators and leaders is important to promote the use of research among clinical nurses. Organisational support is vital to EBP sustainability. When there is no organizational will to perform and support EBP, EBP will not be achieved. Further to this if managers within an institution do not support staff to acknowledge and embrace EBP then EBP cannot be achieved (Shayan et al 2019; Florczak,2016).

One of the major barriers of EBP in this study was that "administration does not support EBP implementation". This finding is reiterated by Renolen et al., (2018), who found that clinical nurses experience a lack of recognition and support from leaders. Hence, they proposed that it is important for leaders to continuously support nurses in their efforts of EBP. Aasekjær et al., (2016) also reported that for an organization's program of EBP to survive long term, leaders should sustain commitment and engagement towards this program. In addition, this study highlighted the top third barrier to research utilization and EBP was nurses not having enough authority to make changes related to patient care. This similar to the findings of Jordan et al., (2016); Baird & Miller (2015); Gerrish & Cooke (2013). However, according is Dunbar et al., (2007) the lack of authority of nurses can be addressed through the development of shared governance systems. Hence nurses and leaders can work together to have one voice in decision making and policy changes that affect patient care and work environments.

A statistically significant difference was noted with age and barriers to research utilization and EBP. Hence, within this study, it can be assumed that the younger nurses reported barriers related to research-related issues, whilst the older nurses reported barriers to shortcomings in themselves as nurses and the organization. This is similar to the findings of Khammamia et al., (2015), who reported that the older respondents in their study reported more organizational barriers than the younger respondents. This finding was attributed to the fact that older individuals might be more aware of the current trends or know that EBP is something that they should be doing. Also, older individuals may be more familiar with hospital systems and factors associated with the use of EBP. Another significant finding within this study shows that the majority of nurses only searched for information, research, or evidence at least 1-2 times a month.

This could be attributed to a lack of time and a lack of skills to search (Bahadori, Raadabadi & Ravangard et al, 2016; Renolen, Høye, Hjälmhult, et al, 2018). According to Majid et al (2011), EBP is a multistep process where nurses need sufficient time for EBP. In addition, Young and Ward (2001) also found that a lack of information skills is a barrier. Further to this, the low use of electronic information sources could be due to a lack of knowledge about the existence of such sources and limited literature searching skills of the nurses (Jones, Schilling and Pesut 2011; Hider et al. 2009).

The findings of this study highlight that the barriers to EBP and research utilization within critical care within Saudi Arabia is similar to barriers faced by health care professionals in developed countries. It is therefore important to support health care professionals who are challenged with limit EBP capabilities. The findings of this study recommend that the phenomenon of barriers to EBP and research utilization be explored from a qualitative lens within a Saudi context to getting a richer understanding of this phenomenon, also, the study can be repeated with a larger sample size in more than one setting.The study limitation included one setting inclusive of just CCNs. In addition, within this study, although the total population was 277 nurses only 96 CCNs participated. This is a response rate of 35% and highlights that nurses are reluctant to partake in research.

CONCLUSION

For EBP and research utilization to become the golden standard of health care, health care organizations and systems should advocate its use. Even though EBP has been highlighted as a core competency for health care professionals, the uptake of EBP into practice remains a challenge.

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Evaluation of Inhibitory Activity of Bacteriocins from *Enterococcus italicus* BLN48 Against *Mycobacterium fortuitum* and its Toxicity Profiling

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ABSTRACT

Lactic Acid Bacteria (LAB) is an important group of microorganism due to their wide application in the food and dairy industries. They have extensively studied for the potential against various bacterial pathogens. The inhibitory activity of LAB is through their production of bacteriocins, organic acids, enzymes, hydrogen peroxide, etc., Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various microorganisms. The incidence of non tuberculous mycobacterial infections increasing worldwide. *Mycobacterium fortuitum* is one of the rapidly growing non tuberculous mycbacteria which causes skin, bone, joint and pulmonary infections. Their antibiotic resistance and prolonged course of treatment necessitates the development of new candidate to fight against them. In this study, we have partially purified bacteriocins from four LAB strains using solvent extraction method and screened their activity against *M. fortuitum* MTCC1902 by colony forming unit (CFU) estimation method. The potential strain was identified by 16S rRNA sequencing and their sequences were submitted to Genbank database. The toxicity of potential LAB strain was assessed by both in vitro and in vivo method against Vero cell lines and zebra fish model respectively.The strain BLN48 showed 97.9% reduction in growth of *M. fortuitum* and 1.89±0.32 log reduction in CFU/ml. The potential strain BLN48 was identified as *Enterococcus italicus*. *E. italicus* BLN48 exhibited cytotoxicity against vero cell lines in dose dependent manner whereas under in vivo conditions, 50% of zebrafish larvae survived upto 144 hours post fertilization (hpf) with normal morphological changes. Further purification and characterization of *E. italicus* BLN48 bacteriocin in future helps in the development of an efficient candidate against *M. fortuitum* as well as other mycobacterial pathogens.

KEY WORDS: PARTIALLY PURIFIED BACTERIOCIN, NON TUBERCULOUS MYCOBACTERIA, MYCOBACTERIUM FORTUITUM, CYTOTOXICITY, ZEBRAFISH.

ARTICLE INFORMATION

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INTRODUCTION

Lactic Acid Bacteria (LAB) remains an industrially important group of microorganism due to their wide application in the food and dairy industries (Gomez et al., 2015). LAB are widely distributed in diverse habitats like marine, food products, dairy origin, etc., Usually, fermented foods were screened for bacterial isolates with antimicrobial properties as its microbiota is dominated by LAB. LAB also exists in gastrointestinal tracts, oral cavities of humans as well as animals (Bungenstock et al., 2020; Li et al., 2020). In raw milk, LAB are the predominant microorganisms and they contribute to the fermentation and food preservation process due to their various metabolite production (Rahmeh et al., 2019). LAB has studied extensively for their antagonistic activity against various bacterial pathogens such as food spoiling microorganisms like *Listeria monocytogenes,* gastrointestinal pathogens and other various gram positive and gram negative bacterial pathogens. Researchers have gained significant attention towards LAB due to their Generally Recognized As Safe (GRAS) status. The inhibitory activity of LAB is mainly through their production of various substances like organic acids, enzymes, bacteriocins, hydrogen peroxide, etc., (Gupta and Garg 2009; Rodrigues et al., 2006 Hussein et al., 2018).

;Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various microorganisms. The uses of bacteriocins in functional foods and as an alternative to antibiotics are their emerging application (Quwehand et al., 2004; Messi et al., 2001). Physical stability and non-toxic nature are the major advantages of bacteriocins (Morgan et al., 2005). Different classes of bacteriocins exerts diverse mechanisms against their target like disruption of cell wall, pore formation, inhibition of protein and nucleic acid synthesis, etc., (Cascales et al., 2007; Stevens et al., 1991). LAB produces a diverse nature of bacteriocins in different size, physicochemical properties, spectrum of activity, structures, etc. They secreted in an extracellular space during LAB growth (Venegas et al., 2019; Anbarasu et al., 2020). The crude and purified bacteriocins from LAB have found their potential use as biopreservative agents to enhance the quality and safety of various food products.The potential bacteriocins can be used in both combined and balanced mode as probiotics for human diseases also (Iseppi et al., 2019, Arrioja et al., 2020).

Non Tuberculous Mycobacteria (NTM) are several mycobacterial species other than *M. tuberculosis* complex and *M. leprae*. NTM causes opportunistic infections in humans as well as animals and also it transmitted among environment, livestock, wildlife, etc. (Odoi et al., 2020). *Mycobacterium fortuitum* is one of the rapid growing NTM and is predominantly found in water systems like natural water, tap water, and water used in showers in hospitals and soil. It mainly causes skin, bone, joint infections and pulmonary diseases in immunocompromised and immunosuppressed patients. They also causes surgical site infections (Okamori et al., 2018; Griffith et al., 2007; Goslee and Wolinsky 1976; Wolinsky and Rynearson 1968; Choudhary et al., 2020).

M. fortuitum are often isolated from skin and soft tissues and also from other clinical samples as it causes many types of infection (Garcia et al., 2020). The development of antibiotic resistance and prolonged course of treatment with multiple antibiotics in NTM infection surges the need for the development of new candidates with potential inhibitory substance and less toxicity to fight against these infections. In this study, we have evaluated bacteriocins from four LAB isolates for their anti *M. fortuitum* activity. The potential LAB isolate was identified through 16S rRNA sequencing and phylogenetic analysis. Toxicity profile of the bacteriocins from the potential LAB strain was also evaluated through in vitro and in vivo method.

MATERIAL AND METHODS

Mycobacterium fortuitum MTCC1902 strain was purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. de Man Rogosa Sharpe broth (Himedia), Middlebrook 7H9 broth (Himedia), Middlebrook 7H11 Agar (Himedia), Chloroform (SRL), PBS tablets (Sigma) were used in the study. Four lactic acid bacterial strains viz., BLN 34, BLN 36, BLN 39 and BLN 48 previously isolated from different cow milk samples were used in this study. Viability of all the cultures were maintained in MRS agar slants at 4°C (Revathy et al., 2019). Bacteriocin from four selected cultures was produced by submerged fermentation process and was partially purified by solvent extraction method using chloroform as described by Burianek et al., 2000 with few modifications. Briefly, 5ml of overnight grown culture in MRS broth was added to 500ml of sterile MRS broth and incubated for 18 hours in shaking incubator at 30˚C. After incubation, the culture was centrifuged at 5000rpm for 10minutes to collect the cell free supernatant. For the extraction of crude bacteriocin, 50% v/v of chloroform has been added to the supernatant and kept in magnetic stirrer at 1000rpm for 20minutes. Then the mixture was subjected to centrifugation at 10000rpm for 30 minutes. After centrifugation, the precipitate in the interphase layer between solvent and aqueous phase was collected carefully and freezed at -20˚C following by lyophilization. The lyophilized form of partially purified bacteriocin (PPB) were stored at -20˚C and used for further assays.

Evaluation of PPB against *M. fortuitum:* Ten mg concentration of partially purified bacteriocin in the form of lyophilized powder was dissolved in 1ml of PBS buffer in order to get 10mg/ml (w/v)concentration. Desired working concentration of PPB was prepared from the main stock using PBS buffer. Inhibitory activity of PPB prepared from all the four cultures was evaluated against *M. fortuitum* by colony forming unit (CFU) estimation (Gillespie et al. 2005).Briefly, *M. fortuitum* MTCC1902 suspension was prepared by inoculating a loopful of *M. fortuitum* culture into 0.3ml of Middlebrook 7H9

broth followed by vortexing. Then the volume of the suspension was made upto 5ml. using 7H9 broth. In a sterile cryovial, 400µl of 7H9 broth was used as growth control and 350µl of 7H9 broth with 50µl of bacteriocin was used in test vial. All the vials were added with 100µl of *M. fortuitum* suspension and incubated at 37˚C for 48 hours. After incubation, 100µl of aliquot from each vial was serially diluted in 900µl of PBS buffer upto 10-12 dilution. 50µl of the dilution was spreaded onto Middlebrook 7H11 agar plate. Plating was done in triplicate and all the plates were incubated at 37°C for 48 hours. The plates with individual colonies were taken for the calculation of CFU/ml.

Taxonomy of potential LAB strain: The genomic DNA of LAB strain BLN48 was isolated using solute ready genomic DNA kit. DNA was analyzed by gel electrophoresis and quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Gloucester, UK). The 16S rRNA gene sequence of the strain was amplified using the primers: 27F 5′AGAGTTTGATCMTGGCTCAG3′ (forward) and 1492R 5′TACGGYTACCTTGTTACGACTT3′ (reverse) (Kumar Gothwal et al., 2007). The PCR amplified product of the strain was sequenced and analyzed at National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (CSIR-NCL), Pune, India. The 16S rRNA gene sequence obtained from the strain BLN48 was aligned with similar sequences available in GenBank using MEGA 7 program. The aligned sequences of the strain BLN48 was used to construct the phylogenetic tree by following neighbor joining algorithm in MEGA 7 program (Saitou and Nei, 1987). The bootstrap estimation (Felsenstein, 1985) was used to determine the confidence of the branches of the phylogenetic tree. The partial 16S rRNA nucleotide sequence of all the four strains has been deposited in GenBank database.

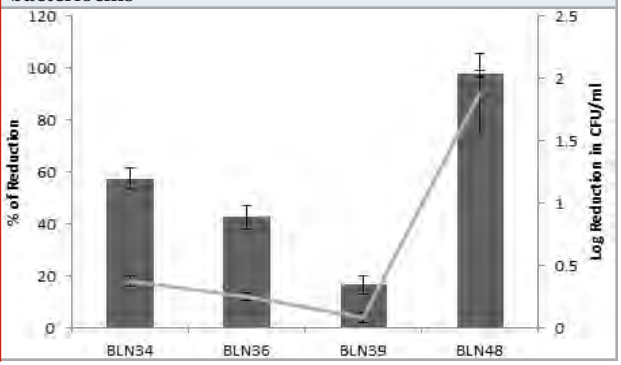
In vitro toxicity analysis of PPB: The cytotoxicity of potential PPB which showed activity against *M. fortuitumwere* assessed in vitro by adopting MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay using Vero cell lines. Briefly, in the 96 well plate, 100µl of RMPI 1640 medium was added with 100µl of desired concentration of PPB. Then 200µl of total volume is gently mixed well. 100µl of diluted PPB from the first well was serially diluted in next well till reaching the lowest concentration. The cultured Vero cell lines were harvested by trypsinization and pooled in 50ml vial. Then the cells were plated at a density of 1x100cells/ml. 200µl of vero cells without PPB was used as a control. The cells were incubated at 37˚C in 5% CO2 incubator for 24 hours. After incubation, 20µl of MTT solution was added to all the wells and incubated for 4 hours at 37˚C.The media and MTT was well mixed and the absorbance was measured at 450nm and the percentage of viability was calculated manually (Vijayarathna and Sasidharan, 2012).

In vivo toxicity analysis of PPB: In vivo toxicity of PPB was evaluated using zebrafish as a model (Sisman et al. 2008). Zero day old zebrafish eggs were purchased from zebrafish aquarium in Kanchipuram district, Tamil Nadu, India. Twenty healthy post hatched zebra fish eggs were transferred to the wells of a 24-well plate along with 1 ml of embryo water (60 mg of sea salt/ litre of ultrapure water). Different concentrations of PPB of potential LAB strain BLN48 (10, 50 and100 µg/ml) was added to the wells and incubated for 144 h at 28.5°C. Mortality of the zebra fish was noted after 24, 48, 96 and 144 h. The embryos appeared opaque and white in colour.The dead embryos were degraded soon, whereas the structures of intact embryos were more visible by 48 hours post fertilization (hpf) which allowed a clear distinction between the dead and alive.The mortality rate is calculated. At the end of the incubation period, the embryos were photographed using a light microscope at 10X magnification.

RESULTS AND DISCUSSION

Anti M. *fortuitum* **activity:** Among the four isolates tested, BLN48 showed significant inhibitory activity against *M. fortuitum* MTCC1902 viz., 1.89 log reduction of CFU/ml which corresponds to 97.9% reduction in growth when compared to growth control. Followed by, BLN34 showed slight inhibitory activity by 0.38 log reduction of CFU/ml with 57.56% reduction from growth control (Figure 1).

Figure 1: Anti *M. fortuitum* activity of Partially purified bacteriocins



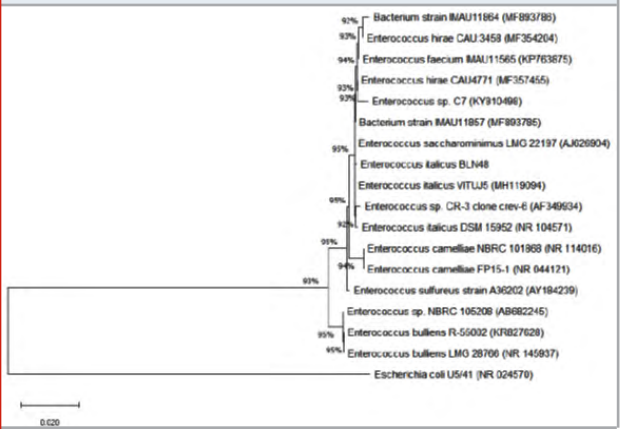
Taxonomy of potential LAB strain BLN48: Amplification of 16S rRNA gene from the strain BLN48 resulted in 1435 bp sequences. BLAST analysis showed 99.43% sequence similarity with 16S rRNA gene sequence of *Enterococcus italicus* DSM 15952. The phylogenetic tree also showed that the strain BLN48 is closely related to *Enterococcus italicus* (Figure 2). The nucleotide sequence of *E. italicus* BLN48 was submitted to Genbank with accession number MN880432.

Table 1: In vitro cytotoxicity analysis of BLN48 by MTT assay against vero cell lines

PPB	Test concentration	Percentage of Viability
BLN48	100mM	16.6
	10mM	60.4

In vitro toxicity analysis of PPB: The cytotoxicity analysis by MTT assay showed that the bacteriocin from BLN48 exhibited the cytotoxicity on vero cell lines in dose dependent manner. At the maximum of 60% of vero cells survived when treated at 10mM concentration of bacteriocin (Table 1).

Figure 2: Phylogeny of BLN48



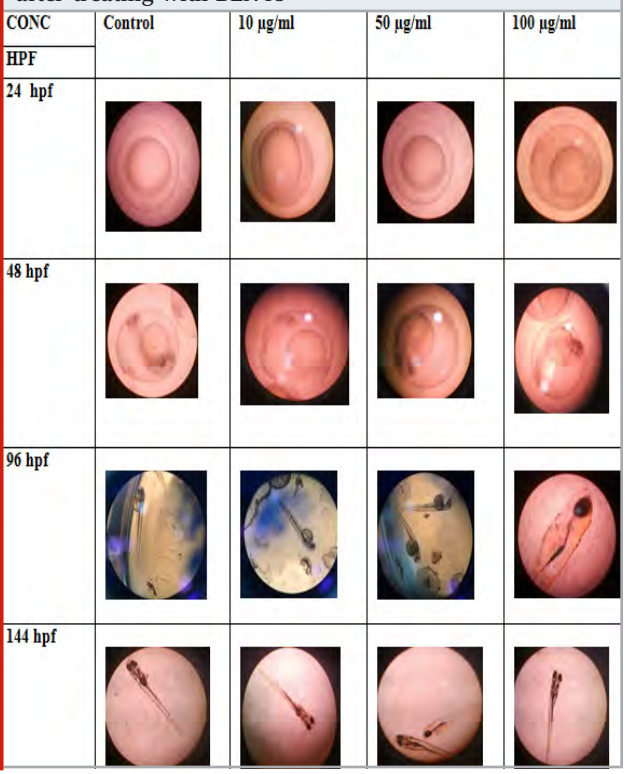
In vivo toxicity analysis of PPB: In the in vivo toxicity analysis of BLN48 done with zebrafish larvae, BLN48 have shown less toxicity viz., around 50% of larvae (compared to control) were survived upto 144 hpf at high concentration (100µg/ml) with healthy morphology under microscopic observation (Table 2). The features like fin movement, swimming nature, tail development are normal to the viable larvae (Figure 3).

Table 2. Mortality rate in in vivo toxicity analysis of BLN48

Concentration HPF	Control	10 µg/ml	50 µg/ml	100 µg/ml
0 HPF	20	20	20	20
24 HPF	20	18	19	16
48 HPF	18	17	15	13
96 HPF	16	14	12	9
144 HPF	13	11	9	7

M. fortuitum group is responsible for 60-80% of post surgical infections caused by mycobacteria. The successful treatment outcome of *M. fortuitum* infections often limited by the multi drug resistance, need of combination therapy, prolonged course, etc. (Cynamon et al., 2012).Santos et al (2016) analysed the resistant profile of *M. fortuitum* isolates and showed their resistance to different classes of antibiotics. Antimicrobial peptides i.e., bacteriocins offers a solution to combat antibiotic resistance of various pathogenic microorganisms. Numerous studies have proved the efficacy of bacteriocins against various drug resistant pathogens (Regmi et al., 2017).

Figure 3: Morphological changes in zebrafish embryos after treating with BLN48



In the present study, the partially purified bacteriocin from *Enterococcus italicus* BLN48 has showed significant inhibition against *M. fortuitum*. In the various studies, numerous antibiotics and chemical compounds have been screened against *M. fortuitum* but there are very less reports on the screening of natural compounds of microbial source against *M. fortuitum* (Gay et al., 1984; Welch et al., 1979; Bagchi et al., 2007). While there are many other reports focuses on the screening of bacteriocins against other mycobacterium species like *M. tuberculosis*. A study by Sosunov et al (2007) has assessed the antimycobacterial of five bacteriocins against *M. tuberculosis* strains. In 2010, Carroll et al., compared the activities of two bacteriocins Lacticin 3147 and nisin against NTM species like *M. kansasii, M. avium* paratuberculosis.

Their study found that lacticin 3147 showed superior activity than nisin. A study by Aguilar-Pérez et al (2018) have found the inhibitory activity of bacteriocin AS-48 against *M. fortuitum* and found their MIC as 64µg/ml along with other mycobacterium species. They also found that there is no cytotoxicity obtained against various macrophage cell lines. Our result showed that the *E. italicus* BLN48 exhibits cytotoxicity on vero cell lines in dose dependent manner. However, the in vivo cytotoxicity assay using zebrafish showed that there is no side effect on the morphology of viable larvae in the presence of *E. italicus* BLN48 at three different concentrations. A survey by Fortina et al (2008) has described the safety and biotechnological properties of *E. italicus* of dairy origin. They suggested that *E.*

italicus presence in the cheese lowers the health risk and supports their applications in dairy industry. This study describes the safety profile of *E. italicus* for their wide application in future.

CONCLUSION

Mycobacterium fortuitum is one of the clinically significant rapidly growing mycobacteria which cause pulmonary, skin and soft tissue infections in immunocompromised and immunosuppressed patients. Partially purified bacteriocin of *Enterococcus italicus* BLN48 showed significant inhibitory activity against *M. fortuitum*. Their cytotoxicity assay under both in vitro and in vivo conditions shows that *E. italicus* BLN48 can be developed as potential candidate against *M. fortuitum*. Further analysis on their Minimum Inhibitory Concentration (MIC), purification and their characterization and screening them against other mycobacterial species will leads to the efficient use of *E. italicus* BLN48 for therapeutic purpose.

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Utilization of Social Media in Orthodontic Practice: Practitioner's Perspective

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ABSTRACT

The objectives of this study were to assess how social media is being utilized by orthodontists, examine orthodontists' preferences regarding social media sites and apps, and to investigate the potential benefits of Internet-based social media sites in improving patient motivation and co-operation, and in enhancing the marketing and communication strategies of orthodontic practices among orthodontists in Riyadh and other cities of Saudi Arabia. Cross-sectional study was performed by utilizing survey research techniques. The participants in this study included orthodontists currently working in private and government dental hospitals in Saudi Arabia. Participants were asked to answer a questionnaire related to their use of social media platforms as a marketing and educational tools in their orthodontic practices. The objectives were thoroughly explained to all participants and an informed consent form was obtained. 31% of orthodontists used social media in their practice while the majority (69%) are not using any form of social media. The highest use of social networking sites were found among those practicing for 6-10 years (40%), the percentage decreases with the age increase. The most commonly used social media platforms among Saudi orthodontists was Instagram (34.6%), followed by Twitter (18.8%). Moreover, the particular purpose for using social media was for education purposes (41%). In addition, there was a reported increase in patients' flow and monthly income among orthodontists using social media in Riyadh and other cities (40.4% and 61.5%, respectively).Most orthodontists have used social media for education and communication purposes. Even though social media should be used to its full capacity as a marketing tool for orthodontists in order to market new materials and techniques to their patients instead of using traditional means of media advertisement to keep their competitiveness with other practices.

KEY WORDS: EDUCATION, MARKETING, ORTHODONTISTS, ORTHODONTIC PRACTICE, PLATFORMS, SOCIAL MEDIA.

ARTICLE INFORMATION

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INTRODUCTION

In the present great revolution of technology, men and women of all ages and professions are consumed in social media and eager to become meaningful parts of such a revolution. Social media was defined by Kaplan and Haenlein, 2010 as "a group of Internet-based applications that build on the ideological and technological foundations of Web 2.0 and that allow the creation and exchanges of user-generated content" (Kaplan and Haenlein, 2010). The use of social media diverts people away from the traditional ways of communication and, hence; making obtaining information totally dependent on internet based networks (Nelson et al., 2015, Akram and Kumar, 2017). Examples of social media websites that most societies use are Facebook and Twitter, with more than 1.2 billion users worldwide (Shabnoor and Tajinder, 2016). Beyond using social media for mere entertainment, these technologies can be used in businesses where they can provide consumers with a variety of products and further allow for their feedbacks (Andzulis et al., 2012).

They can offer many advantages that are cost effective, cost reducing, efficient, and fast in providing customers with information about products and services. This role of social media was not only limited to the realm of business but was also extended to the field of health care, where an increasing number of people worldwide are using social media applications for health-related issues. The use of social media applications has become popular amongst healthcare professionals to communicate with their patients for instructions, medication prescriptions, and health updates. In addition, health care professionals can conduct campaigns for the promotion of health and behavioral changes (Smailhodzic et al., 2016, De Angelis et al., 2018).

The field of dentistry is of no exception, where social media applications initially used by dental schools to promote courses and communication with students by using YouTube and blogs to enhance classrooms experience and interactive learning. Additionally, social media is widely utilized for advertising by private dental clinics, as well as for sharing of dental research through confidential web engines (Neville and Waylen, 2015). This use of social media in dentistry has become of increasing interest and special importance for field of orthodontic practices. Patients' compliance throughout orthodontic treatment course and effective communications are considered among the most important steps toward achieving favorable treatment outcomes. In this context, a study was conducted to assess the effectiveness of YouTube audiovisual information on orthodontic patients with fixed appliances. On average, the result was positive in showing improvement in the patients' knowledge of the dentition and the appliances, especially when compared with the standard methods of instructions (Al-Silwadi et al., 2015).

However, there has been limited research conducted to further explore the widespread and effectiveness of social media usage in the field of orthodontics and, hence; the aim of this study is to assess how social media is being utilized by orthodontists, examine orthodontists' preferences regarding social media sites and apps, and to investigate the potential benefits of Internet-based social media sites in improving patient motivation and co-operation, and in enhancing the marketing and communication strategies of orthodontic practices in Riyadh and other cities of Saudi Arabia.

MATERIAL AND METHODS

This cross-sectional study was performed by utilizing survey research techniques. The study was approved by the Institutional Review Board (IRB) at the College of Dentistry, King Saud University [E-18-3428], and the objectives were thoroughly explained to all participants and an informed consent form was obtained. A self-administered questionnaire was derived from a previous study conducted in Saudi Arabia and modified according to the present study population and aims (Hamasha et al., 2019).

The questionnaire comprised of two sections; the first section consisted of eight questions related to the demographic information of the participants. The second part of the questionnaire consisted of 16 questions related to the use of social media platforms as a marketing and educational tools. The participants in this study included orthodontists currently working in private and government dental hospitals in Saudi Arabia. General dentists, orthodontic residents, retired orthodontists and students were excluded from the study. A list of orthodontists practicing in Saudi Arabia was obtained from the Saudi Orthodontic Society, and an electronic version of the questionnaire was sent through email to all registered orthodontists in Riyadh and Jeddah for a period between January, 2019 to April, 2019.

A Pilot study consisting of 20 participants was conducted to validate the questionnaire and modifications were done accordingly and was assessed by an orthodontist. The questionnaire was prepared in both an electronic format as well as in a printed form. The data was recorded and analyzed using the SPSS software package (Version 23, SPSS Inc., Chicago, IL, USA). Descriptive statistics were calculated and Data analysis was undertaken using Pearson's Chi-square test to compare the association between utilization of social media as a marketing tool and demographic variables with a p-value set at 0.05 for statistically significant associations.

RESULTS AND DISCUSSION

A total of 251 Orthodontists participated in this study consisting of 138 males and 113 females, with a response rate of 76%. Demographic characteristics of the participants were presented in Table 1. More than half of the participants were in the age group of 31-40 (52.2%) and almost three out of four participants were from the city of Riyadh (73.3%). The private sector was the main practice area of the participants (34.3%). Results

shows that 31% of orthodontists used social media in their practice. Only 52 orthodontists in Riyadh are using social media with no significant difference between gender (p-value = 0.095); however, there was a small but statistically significant difference between male and female orthodontists using social media in other cities (p-value = 0.021) (Table 2).

Table 1. Demographic characteristics of the participants

Socio-demographic Characteristics		N	%
Gender	Male	138	55
	Female	113	45
Age Groups	20 – 30	15	6
	31 – 40	131	52.2
	41 – 50	76	30.3
	51 – 61	26	10.4
	Above 60	3	1.2
Nationality	Saudi	163	65
	Non-Saudi	88	35
Region	Riyadh	184	73.3
	Others	67	26.7
Working Sector	Academic	28	11.1
	Academic, Governmental	18	7.2
	Academic, Private	9	3.6
	Academic, Governmental, Private	31	12.3
	Governmental	58	23.1
	Governmental,	21	8.4
	Private		
	Private	86	34.3
Income per month	Less than 20,000 SR	30	12
	20,000 – 30,000 SR	94	37.4
	31,000 – 40,0000 SR	60	24
	More than 40,000 SR	67	26.6

Furthermore, participants were categorized according to the number of years they have been practicing orthodontics as shown in Figure 1. Thus, those who have been practicing for 6-10 years were the highest group in using social media (40%). On the other hand, orthodontists with an experience of more than 10 years had the highest percentage of not using social media in their practice (42.2%). Table 3 presented the frequency distribution of social media use by orthodontists in their practice in relation to social variables and the impact of this utilization on their dental practice. The main social media platform utilized by the participants was Instagram (34.6%), followed by Twitter (18.8%) and Snapchat (15.7%). Moreover, the particular purpose for using social media was for education purposes (41%).

Nonetheless, the analysis of the data shows that the majority of participants were not using social media in their practices (69%) (Table 2). Figure 2 summarizes the main reasons for not using social media among orthodontists. These reasons were related to time consumption (38.3%), concerns about ethical issues (24.3%), as well lack of technical knowledge necessary for using such applications (18%).

According to the survey, utilizing social media in orthodontic practice showed an increase in patients' flow in Riyadh and other cities (52% and 65.4%, respectively). In addition, there was a reported increase of individual's monthly income among orthodontists using social media in Riyadh and other cities (40.4% and 61.5%, respectively). However, no statistically significant difference between gender within Riyadh and other cities were found with p-value > 0.05 (Table 4). Many of orthodontists participated in the survey claimed that there was a relationship between patients' unrealistic expectations and the use of social media, which was statistically significant (P=0.009, P<0.05) as shown in Table 4.

Table 2. Frequency distribution of the social media use relative to the gender and regions.

	Utilizing social media in practice						
Gender	Yes (%)				Total	No (%)	Total (%)
	Riyadh (%)	P.value	Others	P.value			
Male	35 (67.3%)		17 (32.7%)		52 (37.7%)	86 (62.3%)	138 (55%)
Female	17 (65.4%)	0.095	9 (34.6%)	0.021	26 (23%)	87 (77%)	113 (45%)
Total	52 (28.3)		26 (38.8%)			173 (69%)	251
Overall total		78 (31%)					

As reported in several literatures, social media has become widely utilized in health care related practices for the past few years (Kaplan and Haenlein, 2010, Hamasha et al., 2019, Binalrimal, 2019). The purpose of the present study was to assess how orthodontists use social media, and to investigate the benefits of social media in the marketing and communication strategies of orthodontic practice. Most studies have investigated the use of social media among orthodontic patients (Nelson et al., 2015, Papadimitriou et al., 2020).

However, only limited number of researches dealt with the use of social media in orthodontic practice among orthodontists. This study found that only 31% of orthodontists used social media in their practice while the majority (69%) are not using any form of social media. Similar study was conducted to assess the use of social media among dental practitioners working in Saudi Arabia. The authors found that 52% of dentists were using social media in their practices (Hamasha et al.,2019). Another study conducted on the use of social media among orthodontists within the United States showed that 76% of the participants actually use social media (Nelson et al., 2015). In the present study, the respondents were almost equally males and females with the majority of both not using social media (62% and 77%, respectively).

Figure 1: Frequency distribution of the social media use relative to the number of years in orthodontic practice.

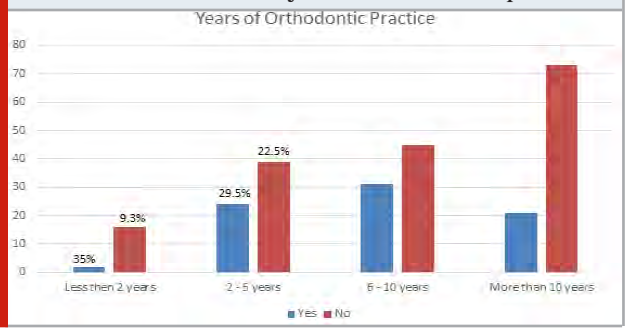
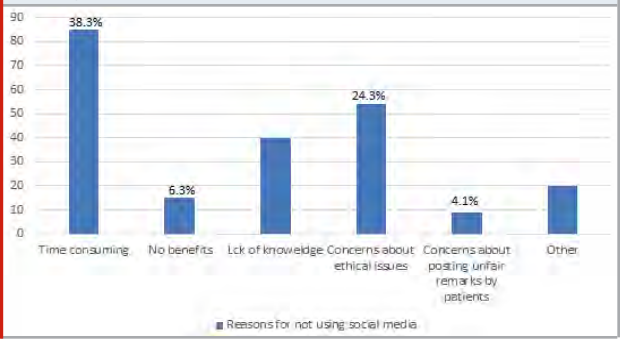


Table 3. The frequency distribution of social media use among orthodontists in their practice.

Variable	Category	No.	%
Social Media Platforms commonly used	Instagram	66	34.6
	Twitter	36	18.8
	Snapchat	30	15.7
	WhatsApp	24	12.6
	Facebook	22	11.5
	YouTube	9	4.8
	Other	2	1
	Blogs	1	0.5
	Telegram	1	0.5
Purposes of Utilizing Social Media	Education	71	41
	Communication	50	29
	Advertisement or marketing	47	27.2
	Other	5	2.8

The respondents using social media in Riyadh city were 28%, with no significant difference between genders, compared to 38% of respondents using social media in other cities within Saudi Arabia, with statistically significant difference between genders. A similar study was conducted to investigate the influence of social media on the perceptions and demands of aesthetic dentistry among the population and dental practitioners as well (Binalrimal, 2019). He attributed the large number of respondents from the central region of Saudi Arabia in populations' and dentists' surveys to the fact that clinicians were adapting their services to meet their patients' demands and inquiries regarding many aesthetic dental procedures, which are more available in large cities than in provisional ones. The discrepancy in this finding with Binalrimal's finding can be potentially attributed to small sample size in this study, as well as, to the fact that other cities included in the present study, such as Jeddah and eastern province are considered as large cities as Riyadh.

Figure 2: The main reasons for not using social media among orthodontists



More than half of the respondents in this study with median age group was 31-40 years followed by median age group of 41-50 years, then there was a decline in the number of the survey participants among older age participants. This study has also found that 40% of the respondents who have been in the practice for 6 to 10 years were the highest group using social networking sites and that usage decreased with age and with those who have been practicing orthodontic for more than 10 years to reach 27%. This decrease supports the finding of several studies who reported that social media usage decreased steadily with age (Nelson et al., 2015, Snyman and Visser, 2014).

In the present study, the most commonly used social media platforms among orthodontists in Saudi Arabia were Instagram (34.6%), followed by Twitter (18.8%). Two different studies among Saudi dental practitioners found that Twitter and Snapchat were the most commonly used social media platforms (Hamasha et al.,2019, Binalrimal, 2019). In contrast, several researchers in the United States found that Facebook was the most utilized social media platform followed by YouTube (Nelson et al., 2015, Snyman and Visser, 2014). The discrepancy in this finding among different studies can be potentially attributed to the sample size, different ethnic backgrounds, and individual's preferences.

In terms of the objectives behind utilizing social media among orthodontists in the present study, it was found that the main objective of using social media was for education purposes (41%), to enhance the learning experience for instance, followed by communication purposes with patients (29%), while using social media

as marketing tool comprised 27% of the responses. Similar findings have reported on faculty members and health disciplines professionals efforts to utilize students' efficiency with social media for educational purposes in order to enhance their teaching techniques, communication, collaboration, and learning experiences, (Kind et al., 2014 Hamasha et al 2019).

This contradicts the finding of several researches who found that the primary use of social media was as a marketing tool and as a means of having a more interactive exchanges with consumers (Nelson et al., 2015, Snyman and Visser, 2014). Further, 38.3% of the respondents in this study attributed the reason for not using social media to the lack of time, pointing to the fact that using networking sites is a time consuming process. On the other hand, 24.3% of the respondents mentioned that the reason for not being active in social media were concerns about ethical issues. This finding is consistent with the conclusion of other researchers, (Binalrimal, 2019, De Angelis et al., 2018). It is also worth noting that, in the field literature, it is argued that behaviors related to professional standards and ethics could present a serious challenge to dental professionals, when using social networking communications (De Angelis et al., 2017).

Table 4. Frequency distribution of social media use among orthodontists in their practice in relation to social variables and its impact on their dental practice.

Variable		Category				
Increased Patients' Flow		Yes (%)	No (%)	I don't know (%)	Total (%)	P-value
Riyadh	Male	19 (54.3)	10 (28.6)	6 (17.1)	35 (100)	
	Female	8 (47.1)	3 (17.6)	6 (35.3)	17 (100)	
	Total	27 (52)	13 (25)	12 (23)	52 (100)	0.317
Others	Male	13 (76.5)	3 (17.6)	1 (5.9)	17 (100)	
	Female	4 (44.4)	1 (11.1)	4 (44.4)	9 (100)	
	Total	17 (65.4)	4 (15.4)	5 (19.2)	26 (100)	
Increased monthly Income						
Riyadh	Male	16 (45.7)	11 (31.4)	8 (22.9)	35 (100)	0.204
	Female	5 (29.4)	4 (23.5)	8 (47.1)	17 (100)	
	Total	21 (40.4)	15 (28.8)	16 (30.8)	52 (100)	
Others	Male	12 (70.6)	1 (5.9)	4 (23.5)	17 (100)	
	Female	4 (44.4)	1 (11.1)	4 (44.4)	9 (100)	
	Total	16 (61.5)	2 (7.7)	8 (30.8)	26 (100)	
Relationship between patients' unrealistic expectations and using social media						
Gender	Male	93 (67.4)	21 (15.2)	24 (17.4)	138 (100)	0.009*
	Female	86 (76)	4 (3.5)	23 (20.5)	113 (100)	
	Total	179 (71.3)	25 (10)	47 (18.7)	251 (100)	

* Statistically significant using Chi-square test

According to orthodontists' perspective surveyed in the present study, utilizing social media in practice increased their patients' flow in Riyadh and other cities. In addition, an increase of individual's monthly income among orthodontists using social media was reported. Similar finding was illustrated by several researchers (Nelson et al., 2015, Huang and Dunbar, 2013) who concluded that orthodontists using social networking sites had more new patients starts per year than those who had not used such sites. Furthermore, many researchers reported the importance of social media usage, emphasizing that dental practices will not survive without a strong online presence (Nelson et al., 2015, Baker, 2012). Many of orthodontists who participated in this study reported that there was a relationship between patients' unrealistic expectations and social media usage, which proved to be statistically significant. This could be explained by the fact that most of what we see on social media simply is not real. Many photo editing and enhancement applications and image manipulation techniques are available on all smartphones and laptops, presenting patients with an unrealistic image of a false sense of the possible changes that can be achieved in real life.

Binalrimal found that 63% of Saudi population believe that social media is not a reliable source of information about aesthetic dentistry due to using photo editing and enhancement programs (Binalrimal, 2019). Therefore, a relationship of trust on social media websites should be established between clinicians and their patients to give them the opportunity to read reviews, leave comments, and provide them with a reliable source of photos and evidence-based information. It is noteworthy that about two-thirds of orthodontic patients, surveyed in a previous study, believed that networking applications on cellphones would help as a reminder for wearing

certain orthodontic appliances, such as orthodontic elastics, functional appliances, etc (Leone et al.,2019). Furthermore, Al-Silwadi et al., in single-centre randomized controlled trial, concluded that presenting an audiovisual information via YouTube website could help orthodontic patients by significantly improving patient knowledge when compared with traditional methods of providing information (Al-Silwadi et al., 2015).

Limitations of the study: The present study has some limitations. Most importantly, a small sample size and sample distribution to represent orthodontists' prevalence in social media usage. Further studies are required to increase the sample size and improve sample distribution to include other regions of Saudi Arabia to investigate the impact of social media in orthodontic marketing strategies. In addition, another study is needed to assess how social media is being used by orthodontic patients seeking support regarding their orthodontic treatment, and looking for an orthodontist with good credentials and expertise with different and new orthodontic procedures.

CONCLUSIONS AND RECOMMENDATIONS

- This study concluded that 31% of the orthodontists in Saudi Arabia use social media in their practice. This percentage could be increased in the future by organizing more workshops and enforcing ethical guidelines for using social media.
- The highest use of social networking sites were found among those practicing for 6-10 years, the percentage decreases with the age increase.
- The most commonly used social media platforms among Saudi orthodontists was Instagram and Twitter.
- Social media use was mostly for education and communication purposes. Even though social media should be used to its full capacity as a marketing tool for orthodontists in order to market new materials and techniques to their patients instead of using traditional means of media advertisement to keep their competitiveness with other practices.

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Solving Student-Project Research Assignment Problems Using a Novel Greedy Linear Heuristic Algorithm: A Case Study at King Saud University, Riyadh Saudi Arabia

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ABSTRACT

There is a growing need to automatically assign project to students due to the increasing number of students. Allocating groups of students to several projects based on predetermined criteria is not a trivial task at most educational institutions. The group of students must enter the score of their project preferences, in the matrix of the project-students matrix. The problem of project-students allocation is becoming more complex and harder when the number of projects and groups becomes bigger. The Graduation Project Committee (GPC) at the Software Engineering Department in King Saud University (SWE-KSU) faces this problem each semester. However, the project allocation process is done at KSU manually. This process is time and effort consuming, especially when dealing with big number of groups. To solve this problem, an automated group-project allocation solver a greedy linear heuristic algorithm namely (GLHA) is proposed. The proposed algorithm finding a student-project optimal stable matching in a sequential liner manner by evaluating project preferences of each group in order to satisfying all the hard constraints (capacity) and the soft constraints (groups' preferences) as much as possible based on the GPA criteria.. As graduation projects for bachelor's degree at KSU-SWE as used a case study. The proposed algorithm GLHA is applied in different KSU-SWE Spring/Fall 2018 real-datasets. The experimental results demonstrate that GLHA has a capability to finds a stable matching of groups to projects when applied to solve the project-group allocation problem at KSU-SWE. GLHA is able efficiently produce a good quality solution in a reasonable period of time. The proposed algorithm is specifically designed to meet the GPC requirements at SWE department. A further work needs to increase the generality of the algorithm to address different type of the project-group allocation problem is further.

KEY WORDS: GROUP-PROJECT ALLOCATION; STUDENT-PROJECT ALLOCATION PROBLEM (SPA), HEURISTIC; GREEDY, ALGORITHM, STUDENT ASSIGNMENT.

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INTRODUCTION

Assigning groups of students to a limited number of projects while satisfying the capacity constraints is a significant chore at the most educational establishments. The group of students must enter score of their project preferences, in matrix of project-students matrix. The problem of project-students allocation is becoming more complex and harder when the number of projects and groups becomes bigger (Budish, & Cantillon,2012). The project conflicts are another issue need to resolved which make the problem of project-students allocation more harder (Arulselvan et. al, 2016). The Graduation Project Committee (GPC) at the Software Engineering Department in King Saud University (SWE-KSU) solve project-students problem every semester. Relegating groups of students to an arrangement of graduation ventures while fulfilling the capacity and GPA criteria for each group. At the moment, the project allocation process is done manually at SWE-KSU by having groups select their list of preferred projects, then having GPC members filter the wishes based on GPA, while making sure the project matches the group's capacity. This process is time and effort consuming, especially when dealing with many projects.

To deal with this issue, the automated group-project allocation solver is proposed in this paper to tackle this problem by satisfying all the constraints. The project-allocation process includes having each group select a list of projects depending on their inclinations and preferences, ordered from the most preferred project to the least preferred project. The group with a higher GPA will be assigned the first choice, if it fulfils capacity constraints. The group-project allocation solver (a greedy linear heuristic) aims to automate the process while satisfying the hard limitations (capacity), GPA, along with the soft limitations (projects preference inclinations).

Related Work: There is a growing need to automatically assign project to students due to the increasing number of students. In the scientific literature, various studies are presented to dealing with the project–students allocation problems. An incredibly old initial study for allocating projects to students is proposed in the 1970s (Proll, 1972). The proposed algorithm was based on a simple objective method for assigning projects to students with consideration of student preferences. A linear-time algorithm is proposed in (Abraham et. al, 2003) to address the student-project allocation problem. The proposed algorithm attempts to find an optimal stable matching with respect to the students' aspects. Kwanashie et. al (2015) proposed an effective method to obtain the a greedy maximum matching assignments based on lexicographically maximum concept. The proposed algorithm is able to find the lexicographically maximum in assignments network.

A recent student-project allocation study has been presented by Chiarandini et. al, (2019) where a mixed integer linear programming problem is formalized to handle the first-year course at the Faculty of Science of the University of Southern Denmark as a case study. The problem is modeled based on model fairness and utilitarian principles. Different state-of-the-art commercial solvers is compared with respect to computational effort and the quality of the allocations solutions by means of a state-of-the-art commercial solver.

The main findings of these models have significant effects on the student assignments distribution. In the Olaosebikan, and Manlove, (2018) model, a student-project allocation problem SPA with lecturer preferences over Students with Ties (SPA-ST) was investigated. The study aimed to find an optimal matching where allocating students over projects based on preferences of student over project. In the work of Abraham et al, (2006), two algorithms were proposed to solve the student-project allocation problem. First algorithm produced best-possible stable matching for students while the second algorithm produced best-possible stable matching for the supervisors. A two sided-based model for the Student-Project Allocation problem was proposed by Manlove & O'Malley, (2008). In this model allocation problems were solved when both students and advisors have preferences over projects.

The main finding of study was that maximum stable matching problem is NP-hard. In (Wilson, 1997) a genetic algorithm with an adapted fitness function was employed to address project assignment problems. The proposed GA based method used dataset of University of Southampton. The experimental result shows acceptable solution could be obtained. Another study (Harper et. al,2005) applied a genetic algorithm to solve the project-students assignment problem. The comparison reveals that the GA based model is superior to the optimal integer programming model and it was able produced a better assignments solution. Many automated matching systems based on efficient algorithms is developed and widely used in various universities such as the University of Southampton (Harper et. al, 2005, Anwar & Bahaj,2003) and University of York (Kazakov, 2002). In (Lightfoot, 2016) an automate system for the assignment of students to projects is developed to find the optimal student-project matching. This system designed to solve the problem of assigning students to projects with consideration only for student preference and capacity as constraints.

None of the previous studies have been addressed student-project allocation problem as we have described in this paper. They had tackled their own student-projects allocations, considering their own criteria. In this paper, we have addressed student-project allocation problems at the Software Engineering Department in King Saud University (SWE-KSU) using a new linear heuristic algorithm that designed to meet the GPC requirements at SWE department. This study is a part of the development of an automate system to be used by the Graduation Project Committee (GPC) at SWE-KSU.

Research Methodology: The project-student assignment problem is a special case of the generalized assignment problem (Harper, et. al,2005, Biró et al 2010). An instance of the project-student assignment problem comprises of a set of project, students, and advisors. Each project should be supervised by only one advisor. The group of project has capacity constraint. Each group of students have preferences over projects. While the advisors have no preferences over the students. The group of students with the higher GPA average is more likely to match with their first preferences. An instance of the project-student assignment problem can be defined as follows: let P = {p1, p2, p3} be a set of projects with its own student capacities, and let G = {G1, G2, G3} be a set of groups. And let AC= {AC1, AC2, AC3} be the set of acceptable project allocation for each group based on capacity (feasible solution).

Figure 1: An instance of the project-student allocation problem

Projects capacity	*Groups with average GPA*	*Groups preferences*
p1: 3 students *p2: 4 students* *p3:4 students*	*g1: 3 students, average GPA_g1= 4.2* *g2= 4 students, average GPA_g2= 3.4* *g3= 4 students average GPA_g3= 4.6*	*g1: {p1, p3, p2}* *g2: {p1, p3, p2}* *g3: {p2, p1, p3}*
	AC1= {p1} AC2= {p2, p3} AC3= {p2, p3} *Assigned Projects= {}*	

Finally, let AG be the set of ordered groups with the highest GPAs. An example the project-student instance is shown in Figure 1. In this example, we have a project capacity of p1 of 3 students, p2 of 4 students and p3 of 4 students. Groups with average GPA as follows: Group 1of 3 students, Group 2 of 4 students and Group 3 of 4 students are having average GPA equal to 4.2, 3.4 and 4.6 respectively. So, the groups preferences are ordering as follows: G1: {p1, p3, p}, G2: {p1, p3, p2} and G3: {p2, p1, p3}. Based on capacity: the acceptable allocation sets for G1 = AC1 , for G2 = AC2 and for G3 = AC3. Thus, AC1= {p1} AC2= {p2, p3} AC3= {p2, p3} with Assigned Projects= {}. Based on GPA: Set of ordered groups with highest GPAs= AG to be: AG= {G3, G1, G2}, start with G3, AG ∩ G3 select first choice = p2 , AG ∩ G1 select first choice = p1, AG ∩ G2 select first choice = p3.

In this section, we propose a greedy linear heuristic algorithm namely (GLHA) to address the project-student allocation problem that described above. The proposed algorithm finding a student-project optimal stable matching in a sequential liner manner by evaluating project preferences of each group in order to satisfying all the hard constraints (capacity) and the soft constraints (groups'preferences) as much as possible based on the GPA criteria. GLHA assigning groups of students over a set of projects through sorting the groups in a descending order based on the average GPA and the project preference of each group is sorted ascending based on the highest weights. To assign a project to a group, the capacity of the project must match the capacity of the group and the project must be available (feasible). The pseudocode of greedy linear heuristic algorithm (GLHA) is shown in Algorithm1.

Initially all group of students in G are unassigned to any project p. Each project p_i has own limited capacity of student. The proposed algorithm begins with sorting the groups ascending based on the average GPA (stept2), then for each group sorting the projects ascending based on the highest group's preference (step3). The group select the project that have the same group capacity, then assign the group\s to the selected project and set the project status as "Taken" and removed from the project set (step9) This process is repeated in step 4 through step 12. The special case for greedy heuristic algorithm is when the two groups have the same GPA and they both select same project; the algorithm will assign the group to the project randomly. The GLHA terminates when all the projects are assigned to groups i.e. the |P| =Ø.

Figure 2

ALGORITHM 1: Greedy Linear Heuristic Algorithm (GLHA)

```
1: procedure GPA (P, G, NG, G_Capacity, P_Capacity, GPA_AVG)
NG is number of group,NP is number of Projects where NP = NG
g ∈ G( group set),p ∈ P (project set)
2:  Sort G ascending based on the GPAAvg
3:  for each g, sort P ascending based on the highest g's preference
4:    for i =1 to NG do
5:          j ←1
6:        do
7:          if (gi capacity == pi capacity)
8:           Assign gi to pi
9:           Remove the pi from P
10:            endif
11:         NP ←NP-1
12:       until (NP ==0)
13:   end for
14: end procedure
```

RESULTS AND DISCUSSION

In this work, we used graduation projects for bachelor's degree at KSU-SWE in the two academic semesters Spring/Fall 2018 as a case study. We applied our proposed greedy linear heuristic algorithm (GLHA) on different KSU-SWE Spring/Fall 2018 real-datasets. The spring semester 2018 dataset, as shown in Table 1, is quite small. It consists only 4 projects and 4 groups with total of students equals to 17. However, the distribution of students among the group is vary between 4-5 students, which make the allocation problems more complex. The fall semester 2018 dataset, as shown in Table 2, it bigger than spring dataset. It consists 13 projects with varied capacities and 13 groups with total of students equals to 65. Despite of the big of dataset, students are distributed among the group are uniformly which make the allocation problems much easier.

We ran GLHA algorithm 10 times for each dataset on Intel(R) Core (TM) and implemented using C# Visual Studio enterprise 2017. For each run, the group's preferences are randomly generated GLHA was able to compute the allocation up to 13 project/group with varied capacities and distribution. Tables 3 and 4 show the results of 10 runs in spring and fall 2018, respectively. For Dataset of spring 2018, the worst obtained solution is the first run as the data set violates the hard constraint (capacity) while the best optimal solution is obtained in seventh run as the dataset as it satisfies the capacity constraint with the less time. For Fall 2018 dataset, the worst obtained solution is the first run where violation of the hard constraint (capacity) is occurs, the best optimal solution is obtained in tenth run as it satisfies the capacity constraint with the less time and it satisfies the soft constraint of groups' preferences.

Table 1. KSU-SWE dataset of Spring2018

groups#	Student Number	GPA Average	Group Preferences	Project #	Project Capacity	Manual Assignments
G1	4	4.50	P2, P3, P1, P4	P1	4,5	G1->P2
G2	4	4.24	P2, P1, P3, P4	P2	3,4	G2->P4
G3	4	4.65	P3, P4, P2, P3	P3	5	G3->P3
G4	5	4.39	P2, P1, P3, P4	P4	4	G4->P1

Total number of groups: 5
Total number of projects: 5
Total number of students: 17
Assignments Time: ≈15 (mins)

Table 2. KSU-SWE dataset of Fall 2018

groups#	Student Number	GPA Average	Group Preferences	Project #	Project Capacity	Manual Assignments
G1	5	4.10	P2, P1, P11, P3,P5, P4, P9, P12, P8,P7,P6,P13,P10	P1	4,5	G1->P11
G2	5	3.99	P2, P1, P3, P8,P9, P4, P5, P6, P7,P11,P12,P13,P10	P2	3-5	G2->P9
G3	5	4.23	P2, P4, P5, P6,P3, P1, P11, P13, P12,P7,P8,P9,P10	P3	5	G3->P4
G4	5	3.50	P3, P2, P4, P1,P7, P6, P11, P12, P10,P8,P5,P9,P13	P4	4	G4->P10
G5	5	4.50	P3, P2, P1, P7,P8, P9, P5, P6, P4,P10,P12,P11,P13	P5	4,5	G5->P7
G6	5	4.89	P2, P3, P4, P1,P6, P5, P7, P8, P10,P12,P9,P13,P11	P6	4,5	G6->P2
G7	5	4.71	P2, P1, P4, P3,P7, P12, P6, P10, P8,P5,P11,P9,P13	P7	4,5	G7->P1
G8	5	4.20	P2, P7, P1, P3,P4, P12, P13, P11, P6,P9,P5,P8,P10	P8	4,5	G8->P12
G9	5	4.42	P2, P8, P6, P5,P9, P7, P4, P1, P3,P12,P11,P13,P10	P9	4,5	G9->P1
G10	5	4.03	P3, P1, P4, P5,P6, P12, P2, P10, P8,P13,P7,P9,P11	P10	4	G10->P6
G11	5	4.66	P5, P1, P2, P3,P4, P7, P8, P12, P6,P10,P11,P13,P9	P11	5	G11->P5
G12	5	4.58	P1, P3, P2, P4,P10, P6, P7, P5, P10,P13,P11,P9,P8	P12	5	G12->P3
G13	5	3.2	P4, P3, P2, P1,P8, P7, P6, P12, P5,P9,P10,P11,P13	P13	5	G13->P13

Total number of groups: 13
Total number of projects: 13
Total number of students: 65
Assignments Time: ≈65 (mins)

Based on the experimental results that shown in tables 2 and 3. We can demonstrate that GLHA has a capability to finds a stable matching of groups to projects when applied to solve the project-group allocation problem at KSU-SWE. GLHA can assigned up to 13 project/group with varied capacities and students' distribution in the most cases with no violation of hard nor soft constraints. the capacity and groups preferences constraints in a reasonable time comparing to the manual assignments. Our proposed algorithm is satisfied both capacity and group's preference constraints. For spring 2018 dataset, the algorithm could meet the group's preference constraints and find the optimal matching in seven out of ten cases. While the algorithm could meet the soft constraints and find the optimal matching in the nine out of ten cases for spring 2018 dataset. It is interesting the GLHA perform slightly better in the fall 2018 dataset than the spring 2018 dataset. This can be indicating that

the GLHA can be more efficient when solving a dataset with bigger size.

Table 3. Experimental results on Spring 2018 KSU-SWE dataset

Run	#Groups/ Projects	Use of Capacity	Violates Capacity Hard Constraint	Violates Group/s Preference Soft Constraint	Time (sec.)
1	4	Yes	Yes	Yes	1.55
2	4	No	No	Yes	1.37
3	4	Yes	No	No	1.15
4	4	Yes	No	No	1.18
5	4	Yes	No	No	0.58
6	4	Yes	No	Yes	1.45
7	3	No	No	Yes	0.43
8	3	Yes	No	No	0.59
9	3	Yes	No	No	0.55
10	3	Yes	No	No	0.54
Best	7	-	-	-	0.43
Worst	1	-	-	-	1.55

Table 4. Experimental results on fall 2018 KSU-SWE dataset

Run	#groups/ projects	Use of capacity	Violates Capacity Hard Constraint	Violates Group/s Preference Soft Constraint	Time (sec.)
1	13	No	No	Yes	2.75
2	13	Yes	Yes	Yes	2.35
3	13	Yes	No	No	2.27
4	13	Yes	No	No	2.20
5	12	No	No	No	1.27
6	11	Yes	No	No	1.45
7	12	Yes	No	No	2.43
8	10	Yes	No	No	1.75
9	11	Yes	No	No	2.36
10	9	Yes	No	No	1.25
Best	10	-	-		1.25
Worst	2	-	-		2.35

The distribution of student over the groups can be an effective factor. As students are distributed uniformly in the fall 2018 dataset which might affect the performance of the GLHA positively. It is It is known that the number of students in the groups are strongly related the project capacity (soft constraint). This is so clear in the result of the spring 2018 dataset. The violation that occurs in some case belongs to the variance of the student's distribution and projects capacities. No existing studies are addressed same SWE_KSU student-project allocation problem with same constraints and criteria. However, Our proposed GLHA could be comparative to the method presented in (Chiarandini et. al, 2019) that addressed student-project allocation problem for the Faculty of Science of the University of Southern Denmark in team of the performance and speed. Our method are able to perform the allocation process in the responsible time with good quality solution). However We believe that the complexity of the dataset might be challenging to the GLHA in some cases. we strive to increase the generality of the algorithm to address different type of the project-group allocation problem in the future. Also, more enhancements will be added to the greedy linear heuristic algorithm to increase its efficiency.

CONCLUSION AND FUTURE WORK

In this paper, a greedy linear heuristic algorithm (GLHA) is presented to solve group-project allocation problem, by satisfying all the hard constraint (capacity) and the soft constraints (groups' preferences) as much as possible based on the GPA criteria. The aim of our proposed heuristic is to produce a good quality solution in a reasonable time. Moreover, the paper presents the project-group allocation problem at SWE-KSU using a greedy linear heuristic algorithm. The proposed algorithm is specifically designed to meet the GPC requirements at Software Engineering department at KSU. However, we strive to increase the generality of the algorithm to address different type of the project-group allocation problem in the future. Also, more enhancements will be added to the greedy linear heuristic algorithm to increase its efficiency.

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The Severity of the Disaggregation Function of Blood Vessels in Piglets of Plant Nutrition

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ABSTRACT

It becomes clear that to ensure homeostasis in piglets during their early ontogenesis, the functional parameters of platelets and vascular walls are of great importance. The level of their hemostatic capabilities in piglets of any age provides the level of functional readiness of primary hemostasis, the degree of perfusion of blood through tissues and the severity of anabolism in them. These circumstances give reason to believe that the severity of platelet aggregation and vascular disaggregation capabilities and their ratio during their growth and development very significantly affect the dynamics of body weight of piglets, that is, on economically important signs. In the blood of piglets during the phase of plant nutrition, the study found a decrease in peroxidation due to the strengthening of the antioxidant properties of their plasma. At the same time, at the end of early ontogeny, piglets showed low platelet activity and pronounced vascular disaggregation capabilities. The dynamics of platelet aggregation capabilities found in piglets during the phase of plant nutrition was fully compensated by changes in the severity of the disaggregation function of the walls of their vessels..

KEY WORDS: PIGLETS, EARLY ONTOGENESIS, PHASE OF PLANT NUTRITION, BLOOD VESSELS, PLATELETS, AGGREGATION, DISAGGREGATION.

INTRODUCTION

Modern pig farming is a high – tech agricultural industry can in the short term to provide the population with protein food in many countries (Maksimov et al., 2018). It provided for a large growth rate of pigs, their high fertility. For this reason, the pig is a highly profitable industry, attractive for investment. The increase in population around the world poses to agriculture the task of increasing the volume of high-quality protein products that can be achieved is largely due to additional intensification of pig production, based on introduction in practice of new knowledge on the physiology of piglets. More relevant in this regard are the data on dynamics in pigs especially hematological and hemostatic parameters in the course of their growth and development (Tkacheva and Zavalishina, 2018). A very important part of hemostasis, including in growing piglets, considered to be the walls of blood vessels and platelets (Zavalishina, 2018a; Zavalishina, 2018b).

Their hemostatic properties determined in these animals the work of the entire primary hemostasis and intensity of microcirculation in all organs (Bikbulatova, 2018a; Zavalishina, 2018c), creating conditions for anabolism output on the adult level for all parameters (Zavalishina, 2018d). Apparently, the activity of platelet aggregation

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and disaggregative influences on it of the vessels at the final stage of early ontogenesis is associated with the growth intensity of piglets, and hence affects the development of their productive parameters (Korepanova et al., 2015). However, the severity of the ability of platelets to aggregate and disaggregative capabilities of the vessel walls in healthy piglets at the end of the early ontogeny remain poorly studied. In the work goal: to consider the characteristics of platelet aggregation and disaggregative influences from the vessels in piglets during the phase of plant food.

Table 1. Indicators of primary hemostasis in piglets of plant nutrition.

Indicators	Age of piglets, n=32, M±m				
	41 days	90 days	150 days	200 days	230 days
Platelet aggregation s with ADP,	34.1 ±0.12	32.2±0.10	30.1±0.14 p<0.05	28.2±0.10 p<0.01	25.7±0.08 p<0.01
Vascular wall anti-aggregation index with ADP	1.49±0.07	1.53±0.07	1.57±0.06	1.61±0.07	1.66±0.04 p<0.05
Platelet aggregation with collagen, s	23.6±0.08	21.5±0.12	20.0±0.15 p<0.05	18.7±0.06 p<0.01	15.9±0.07 p<0.01
Collagen vascular wall anti-aggregation index	1.51±0.04	1.53±0.05	1.56±0.08	1.60±0.07	1.65±0.03 p<0.05
Platelet aggregation with thrombin, s	49.0±0.10	47.2±0.14	44.3±0.05 p<0.05	41.3±0.11 p<0.01	38.4±0.04 p<0.01
Vascular wall antiaggregation index with thrombin	1.53±0.08	1.55±0.08	1.58±0.17	1.62±0.05	1.67±0.06 p<0.05
Platelet aggregation with ristomycin, s	34.9±0.14	32.1±0.06	29.8±0.19 p<0.05	26.9±0.14 p<0.01	24.3±0.09 p<0.01
Vascular wall antiaggregation index with ristomycin	1.54±0.02	1.57±0.05	1.61±0.08	1.64±0.06	1.69±0.05 p<0.05
Platelet aggregation with H_2O_2, s	36.5±0.13	34.0±0.18	31.6±0.10 p<0.05	28.2±0.08 p<0.01	25.5±0.12 p<0.01
Vascular wall anti-aggregation activity index with H_2O_2,	1.53±0.07	1.55±0.06	1.58±0.06	1.63±0.10	1.68±0.03 p<0.05
Platelet aggregation with adrenaline, s	85.6±0.15	83.2±0.18	78.5±0.17 p<0.05	75.2±0.13 p<0.05	71.4±0.10 p<0.01
Adrenaline vascular wall anti-aggregation index	1.56±0.08	1.58±0.07	1.61±0.05	1.64±0.06	1.70±0.09 p<0.05
Platelet count in aggregates, %	8.7±0.09	9.0±0.06	9.4±0.10 p<0.05	9.8±0.05 p<0.05	11.5±0.03 p<0.01
The number of small aggregates of 2-3 platelets per 100 free-lying platelets	4.2±0.09	4.5±0.05	4.9±0.08 p<0.05	5.5±0.04 p<0.01	6.1±0.05 p<0.01
The number of medium and large aggregates of 4 or more platelets, per 100 free-lying platelets	0.26±0.05	0.28±0.03	0.31±0.05 p<0.01	0.34±0.07 p<0.01	0.37±0.03 p<0.01

MATERIAL AND METHODS

The work was carried out in accordance with the ethical standards outlined in the European Convention for the Protection of Vertebrates Used for Any Scientific Purpose. This convention was adopted in Strasbourg on March 18, 1986 and reaffirmed in Strasbourg on June 15, 2006. For the study, 32 pigs of large white breed aged plant nutrition were taken under observation. All animals were observed daily throughout the study period. They were examined using the following list of methods 5 times: at the age of 41 days, at the age of 90 days, at the age of 150 days, at the age of 200 days, and at the age of 230 days of life.The piglets taken into the work recorded blood concentrations of acyl hydroperoxides and products capable of reacting with thiobarbituric acid using a set of reagents manufactured by Agat-Med (Russia). The amount of plasma antioxidant activity was found out in the piglets taken into work (Barkagan and Momot, 2008).

Using the method of visual assessment of the development of platelet aggregation (AP) (Shitikova, 2000), a standard set of inductors was used in their standard plasma

concentration, which was previously strictly adjusted to the standard level of platelets in it. To assess the disaggregation properties of blood vessels, the value of the index of antiplatelet activity of the vascular wall was calculated. This was accomplished by dividing the value of AP in plasma, which was taken under conditions of temporary venous occlusion of a vein, by AP in plasma obtained without applying a tourniquet to a vein (Shitikova, 2000). The intensity of platelet aggregation occurring in blood in vivo was recorded using phase contrast studies (Shitikova, 2008). The data obtained in the study, processed by the standard criterion (td) of student.

RESULTS

During the age in question, piglets had a gradual decrease in the concentration of acyl hydroperoxides from 1.38±0.016 D233/1 ml to 1.23±0.019 D233/1 ml and products capable of reacting with thiobarbituric acid from 3.25±0.031 mmol/l to 2.99±0.022 μmol/l, respectively. The found changes are based on the increase in the observed plasma level of plasma protection from 36.2±0.19% to 39.8±0.11%. During the phase of plant nutrition in the examined piglets, a gradual acceleration of antibodies was recorded. Most rapidly, their AP occurred in response to the addition of collagen (table1). Slightly slower than AP occurred under the influence of ADP, ristomycin and H_2O_2. The use of thrombin and adrenaline as an inducer led to the development of their antibodies at an even later date.

During the observation period, piglets showed an increase in the values of the indices of the antiaggregatory activity of the vascular wall with respect to all applied inducers (table 1). The maximum value was the index of anti-aggregation activity of the vascular wall with adrenaline due to the most pronounced inhibition of antibodies caused by this inducer in plasma obtained from blood after application of the cuff to the vessel. Slightly lower in the observed animals was this index for H_2O_2 and ristomycin. Even lower is this indicator in terms of collagen (at the end of observation 1.65±0.03), in terms of ADP (at the end of observation 1.66±0.03) and in terms of thrombin (at the end of observation 1.67±0.06).

During the phase of plant nutrition in the blood of piglets, an increase was found in the number of aggregates having a small size by 45.2%, as well as aggregates with a medium and large size by 42.3%. During their observation, the number of platelets in the aggregates up to the level of 11.5±0.09% also increased in their blood (table 1).Thus, in piglets, the normal course of the last phase of early ontogenesis increases the power of the disaggregation effects of blood vessels on platelets, which should be considered as a serious mechanism for providing homeostasis in the whole body by ensuring the normal functioning of the primary hemostasis.

The final phase of early ontogenesis in pigs is marked by the process of steady maturation of organs with the achievement of adequate adaptation to existing conditions (Bekenev, 2012). In this period, piglets are preparing all cells for adulthood and all internal organs are strengthened (Vorobyeva et al., 2018). A blood system and its subsystem, hemostasis, are considered to be a particularly significant system that preserves the optimum functioning of the body (Zavalishina, 2018e). The work of the latter goes through several processes and implements preservation of blood flow during hemocirculation and thrombosis after vascular damage (Zavalishina, 2020a). The depression of the severity of plasma peroxidation found in the examined piglets ensured the morphological integrity of platelets, thereby limiting their activity. This is very strongly determined by the gradual production of thromboxane in their platelets. This leads to better hemocirculation in all tissues, adequate to the needs of the body at the end of early ontogenesis (Zavalishina, 2018f; Karpov et al., 2020).

The activation of the adhesive properties of platelets in animals revealed during the study during the observation period was due to an increase in the density of collagen receptors on them. The presence of amplification of this process was confirmed in piglets by the development of AP acceleration with collagen inducer (Zaitsev, 2019). Also, in piglets during the course of the observation, platelet adhesion increased, which was largely due to the intensification of the generation of von Willebrand factor molecules in the vessels, which interacts with platelets by connecting with specific platelet receptors (Zavalishina, 2018g; Solovyova et al., 2020).

Strengthening of the adhesive properties of platelets occurred in piglets along with an increase in their aggregation. An increase in the severity of the results of the interaction of platelets with strong inducers of platelet aggregation was manifested due to the stimulation of the phosphoinositol mechanism of the flow of hemostatic manifestations of platelets, the enhancement of the enzymatic properties of phospholipases and the increase in the severity of phospholation of actin and myosin in platelets (Zavalishina, 2020b). The acceleration of the onset of platelet aggregation in response to the action of weak inducers was due to the growth of glycoprotein molecules on the platelet membranes, acting as their receptors (Kiperman, 2010) and the enhancement of platelet enzymes of the thromboxane synthesis system (Zavalishina, 2018h; Krapivina and Kryazhev, 2020).

The study in piglets throughout the observation found increase disaggregative manifestation from the vessels, which was associated with the increased generation in their walls physiologic antiplatelet agents. Due to the upcoming changes of the examined animals developed a biological balance between the degree of aggregation and disaggregation process in the blood. Increase in blood of pigs during the observation of the level of substances antiplatelet agents weakened the implementation of the reception and intracellular mechanisms clumping of platelets. It had its basis in piglets of maintaining a strict ratio of output intensity in the blood of substances with proaggregant and antiplatelet properties, which is very

important for hemostasis (Bikbulatova, 2018b).

For surveillance in pigs found increased aggregation of platelets under conditions of blood flow, which was confirmed by its status in animals in vivo. It is sufficient in the blood of observed pigs were provided increased with the age of numerical values of the index antiaggregatory activity of their vessels adequate increase of platelet aggregation (Zavalishina, 2018i). Optimum of their relation proves the adequacy of the disaggregative effects of vessels on increasing with age in piglets platelet activity during hemocirculation (Zavalishina, 2018j). It is clear that it is a powerful adaptation mechanism for restricting growing pigs at the end of their growth excessive platelet activity, and ensure they have the normal flow of hemocirculation (Lazareva, 2005; Zavalishina, 2018k). Developing a clear correlation proaggregation and antiaggregation processes have observed piglets confirms that they have stability in morphological terms, the endothelial layer of the vessels engaged in the synthesis of substances, limiting activity of platelets (Momot, 2006; Zavalishina, 2018l).

CONCLUSION

A clear balance of vascular and platelet activity in mammals is considered extremely important for hemostasis. The adequacy of platelet processes in growing animals in it directly ensures the functioning of primary hemostasis and supports blood circulation in organs. There is no doubt that these processes strongly affect anabolism at the end of early ontogenesis. In the work done, in piglets at the last stage of their organism formation, a clear correlation of platelet activity and increasing level of antiaggregational properties of blood vessels was recorded. This dynamics was the basis for maintaining the optimum blood rheology in any vessels. The detected platelet activation in piglets during the observation period is fully functionally balanced by an increase in hemostatic manifestations of blood vessels. Maintaining a balance between the level of platelet activity and vascular control over it during the final stage of early ontogenesis should be considered extremely important for the normal growth of piglets to an economically preferred level.

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Effect of *Aloe vera* Ethanol Extract on Lipid Accumulation During Adipocyte Differentiation Using 3T3-L1 Cell Line

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ABSTRACT

For the past few decades, medicinal plants have been inspected to have an anti-obesity effect, including *Aloe vera*. Various of study has been conducted but shows different results regarding the effect of *Aloe vera* on lipid accumulation. *Aloe vera* have showed to be an inhibitory effect in adipogenesis by downregulation of PPAR expression which can suppressed lipid accumulation. This study aims to examine the effect of *Aloe vera* ethanol extract on lipid accumulation during adipocyte differentiation using 3T3-L1 cell line. Four sets of 3T3-L1 preadipocytes along with the *Aloe vera* ethanol extract treatments (0 ppm, 10 ppm, 20 ppm, and 40 ppm) were prepared. Adipogenic differentiation cocktail consisting of 0.5 mM isobutylmethylxanthine (IBMX), 0.25 µM dexamethasone, and 1µg/mL insulin were given to each well on day 0. Mediums were replaced every two days. On day 12, the wells were stained with Oil Red O, and the red-stained lipid droplets were observed under the microscope. Macroscopically, the red stain showed almost the same amount of stain within treatments. The control group and 20 ppm group showed a slight increase in lipid accumulation compared to 10 pp and 40 ppm gorup when observed under the microscope with 40x and 100x magnification. The lipid accumulation were then measured using a spectrophotometer at a wavelength of 550 nm for quantification. The addition of *Aloe vera* ethanol extract in 3T3-L1 preadipocytes showed no significant differences in lipid accumulation ($p>0.05$), although it showed a decrease in the lipid absorbance value. In conclusion, the addition of *Aloe vera* did not reduce the lipid accumulation in 3T3-L1 cell differentiation

KEY WORDS: 3T3-L1 CELL LINE, ADIPOCYTE DIFFERENTIATION, ALOE VERA, LIPID ACCUMULATION.

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INTRODUCTION

Obesity is becoming one of Indonesia's major health issues, and is associated with an elevated mortality risk of cardiovascular diseases (Harbuwono et al, 2018; Jiang et al, 2013). Studies on a vast range of medicinal plants have been investigated and reported to be useful in treating obesity (Hasani-Ranjbar et al, 2013). The researches are focused on searching for herbal plants that can prevent excess body fat accumulation (Misawa et al, 2012). These supplements modify the weight regulation of the human body by altering appetite, metabolism, or absorption of calories (Chandrasekaran et al, 2012).

For thousands of years, *Aloe vera* has been broadly applied as a medicinal plant to overcome various health problems, including obesity (Christaki and Florou-Paneri, 2016; Rajeswari et al, 2012). A previous study examined the effect of Aloe-emodin, an anthraquinone compound isolated from *Aloe vera* leaf, on 3T3-L1 preadipocyte and resulted in suppressed lipid accumulation during the adipocyte differentiation using 3T3-L1 preadipocyte (Anand et al, 2010). The suppressed PPARγ expression is thought to be the reason (Anand et al, 2010). Adipocyte differentiation was also nearly blocked with 20μM *Aloe vera* in a study using human mesenchymal stem cells (Subash-babu and Alshatwi, 2012). Whereas in other studies, the administration of dietary aloe QDM complex in obese mice lowered the body weight and suppressed the expression of PPARγ which then averts the differentiation of adipocyte (Shin et al, 2012).

A recent study showed that oral administration of isolated phytosterols from *Aloe vera* significantly reduced visceral fat weights than obese control in Zucker diabetic fatty rats (Misawa et al, 2012). The antiobesity mechanism in the study suggested that the ingestion of isolated phytosterols of *Aloe vera* suppressed the expression of gluconeogenic and lipogenic enzymes, and enzymes related to glycolysis and lipolysis were elevated. In addition to that, transcriptional factors were also significantly decreased in which can ultimately inhibit adipocyte differentiation (Misawa et al, 2012). However, in other studies, *Aloe vera* supplementation showed an insignificant reduction in body weight gain in high-fat diet (HFD) mice (Pothuraju et al, 2016).

Nevertheless, the effect of *Aloe vera* extract on adipocyte differentiation has not been fully understood. Dysregulation and dysfunctional of adipocyte plays a critical role in the etiopathogenesis of obesity (Unamuno et al, 2018). Adipocytes are formed by the process of preadipocytes proliferation and differentiation (Moreno-Navarrete and Fernández-Real, 2012). Parameters used to assess adipocyte differentiation are lipid accumulation and the increased expression of specific adipocyte genes (Moreno-Navarrete and Fernández-Real, 2012; Ruiz-Ojeda et al, 2016).

As obesity is becoming a major health issues, researchers were also challeged to study vast range of medicinal plants to overcome it, this includes *Aloe vera*. Further studies of the effect of *Aloe vera* ethanol extracts on adipocyte differentiation need to be carried out. Studies using the adipocyte differentiation system is one way that can be done to examine the effects of *Aloe vera* on the development of obesity. This study aims to investigate the effect of *Aloe vera* ethanol extract on adipocyte differentiation, mainly in lipid accumulation, using 3T3-L1 cell lines.

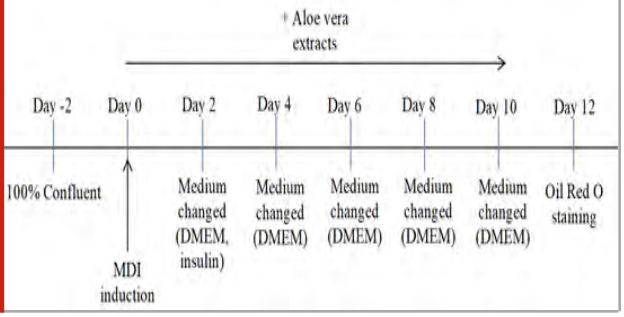
MATERIAL AND METHODS

Research design: This is an experimental analytical qualitative and quantitative study. The qualitative study focused on the morphology of the Oil Red O stained lipid accumulation, whereas the quantitative analysis was carried out to validate the qualitative observation. The objective of this study is to observe the effect of *Aloe vera* ethanol extract in inhibiting lipid accumulation during 3T3-L1 preadipocytes differentiation.

***Aloe vera* ethanol extraction:** *Aloe vera* leaves were purchased from a local supermarket in Bandung. Leaves were washed, peeled, and dried. The dried *Aloe vera* were then blended using an electric blender and boiled with ethanol 95% and later filtered. The filtrate was concentrated, obtaining thick gel-like extract of *Aloe vera*. Before use, the particles were diluted with dimethyl sulfoxide (DMSO) solution to create an extract with a concentration of 10 ppm, 20 ppm, and 40 ppm.

Cell culture and differentiation: The protocol used in this analysis was based on previously published study (Ariyanto et al, 2019). This study was conducted at Cell Culture Laboratory, Faculty of Medicine, Universitas Padjadjaran, Indonesia. Cell culture was the first step in performing this experiment. 3T3-L1 cell lines were cultured in Dulbecco's Modified Edge's Medium (DMEM), obtained from Sigma-Aldrich, containing 10% Fetal Bovine Serum (FBS) and stored at 37˚C, 5% CO. The cells were left to grow for 48 hours, or until it has reached 100% confluent. Lastly, cells were incubated as a confluent culture for another 48 hours (Figure 1).

Figure 1: Cell culture and differentiation protocol. MDI: differentiation cocktail consisting of 0.5 mM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone, and 1 μg/mL insulin; Insulin: 1 μg/mL insulin-containing DMEM.



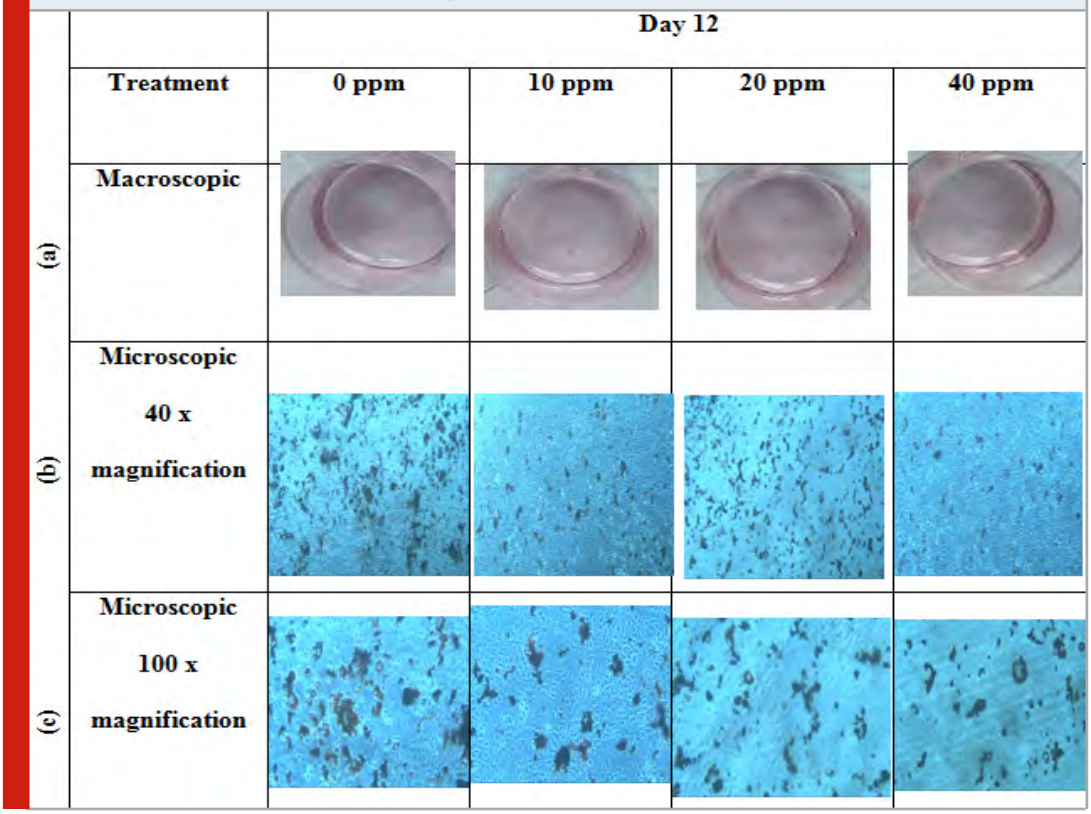
The induction of adipocyte differentiation started right after the incubation process and was marked as day 0. In this experiment, four different mediums were prepared in a twelve-well plate: medium control (0 ppm), medium with 10 ppm *Aloe vera* ethanol extract, medium with 20 ppm *Aloe vera* ethanol extract, and medium with 40 ppm *Aloe vera* ethanol extract. The MDI (0.5 mM IBMX, 0.25 µM dexamethasone, 1µg/mL insulin), obtained from Sigma-Aldrich, cocktail differentiation were also set. On day 0, wells containing 3T3-L1 cell lines were given MDI induction with different extract treatments depending on the dosages. On day 2, the medium of the wells were exchanged DMEM and insulin mediums. As in day 4, the wells were replaced with DMEM only. The medium was continuously changed throughout the experiment every two days until mature adipocyte were obtained. Samples on day 12 were then stained using Oil red O.

Oil Red O staining: Upon completion of the differentiation process, the cells were washed twice with PBS and fixed for 20 minutes with formaldehyde 4%. Isopropanol 60% were given to wash the cells before staining. Oil Red O solution was used to stain the cells for 15 minutes. The wells were rewashed with Isopropanol 60%. Cells were then observed under the microscope. Red stain indicates lipid accumulation.

Quantification: Isopropanol 98% were added to the cells to elute the stain of Oil Red O. Oil Red O absorbance values were determined using a spectrophotometer at wavelength of 550 nm.

Statistical analysis: GraphPad Prism version 8.3 for Windows (GraphPad software, Inc. San Diego, CA) was used to perform statistical analysis. The data obtained were expressed as mean ± standard deviation (SD). Differences between separate treatment variations were analyzed using one-way ANOVA with Tukey's posthoc test. Differences were considered statistically significant if the value of $p < 0.05$.

Figure 2: Macroscopic and microscopic features of Oil Red O stained on 3T3-L1 cells with four different treatments: 0 ppm, 10 ppm, 20 ppm, 40 ppm: (a) macroscopic images, (b) microscopic 40x magnification images, and (c) microscopic 100x magnification images.

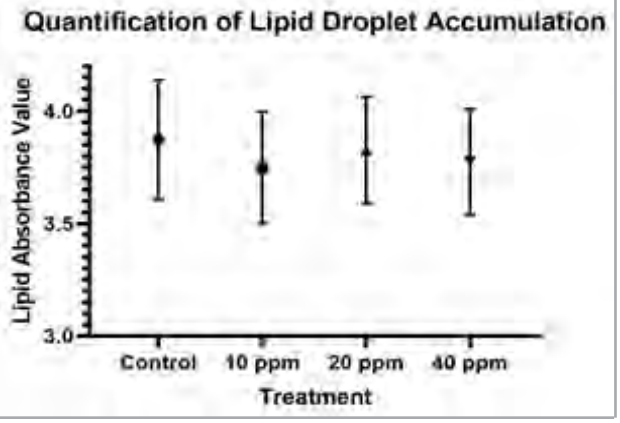


RESULTS AND DISCUSSION

Qualitative analysis of lipid accumulation: Lipid accumulation on each well was observed macroscopically by the naked eye and microscopically under the microscope with 40x and 100x magnification. Macroscopically, the red stain showed the amount of the lipid droplet formation (Figure 2-a). The red stain within each treatment showed almost the same amount of stain, which indicates no significant differences between treatments. Lipid accumulation observed under the microscope with 40x and 100x magnification showed that the control group and 20 ppm group has a slight increase in lipid accumulation compared to 10 ppm and 40 ppm group (Figure 2b-c). Quantification of lipid droplets formation was then counted to confirm the following observation.

Quantitative analysis of lipid accumulation: At the wavelength of 550 nm, the control group turned out to have the highest lipid absorbance value compare to the other groups. However, the higher the extracts given did not show a constant decline in the lipid absorbance value. 10 ppm group showed the lowest lipid absorbance value, whereas 40 ppm group and 20 ppm group became the second and third lowest lipid absorbance value, respectively. Statistically, the outcome of this analysis showed no significant difference between groups, even though the administration of *Aloe vera* ethanol extract showed a decrease in lipid absorbance value ($p>0.05$) (Figure 3).

Figure 3: Quantification analyses of lipid droplets accumulation. Oil Red O stains were eluted with isopropanol 98%. Lipid absorbance values were measured using a spectrophotometer at wavelength 550 nm. ($p>0.05$).



This research investigated the effect of *Aloe vera* ethanol extract on lipid accumulation using 3T3-L1 preadipocytes cell line. The purpose of this study is to investigate whether *Aloe vera* ethanol extract supplementation in 3T3-L1 cell lines can suppress lipid acculumation during adipocyte differentiation. Adipocyte differentiation process requires a complex algorithm of various adipogenic gene expression (Lowe et al, 2011). Cooperative manner of peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAT/enhancer-binding proteins (C/EBPs) gene expression plays a vital role in adipogenesis, which together forms mature adipocyte phenotype (Moseti et al, 2016). In addition to that, sterol regulatory element-binding protein (SREBP) has the ability to induce the expression of PPARγ, which also makes it a key determinant of adipocyte fate (Ruiz-Ojeda et al, 2016; Moseti et al, 2016). Understanding of these processes leads us to a better comprehension of courses that involves the inhibition of adipogenesis.

Various studies on *Aloe vera* effects have been performed. An earlier study reported an insignificant reduction in body weight gain between HFD mice and HFD mice supplemented with *Aloe vera* (Pothuraju et al, 2016). In another study, *Aloe vera* supplementation towards obese mice also exhibited no difference compared to HFD obese mice (Shin et al, 2011). Similarly, *Aloe vera* also did not present a significant difference in body weight reduction in HFD mice in a reasearched carried out by Chihara et al (Chihara et al, 2013).

In addition to that, there are also plenty of studies that exhibit a significant weight loss with the administration of *Aloe vera*. A study showed a decrease in body weight and visceral fat weight in *Aloe vera* gel powder given obese mice compared with controlled obese mice (Misawa et al, 2012). Other prior studies have also shown that oral administration of isolated phytosterols from *Aloe vera* may reduce visceral fat weights significantly compared to obese control in ZDF rats (Misawa et al, 2012). SREBP1, a pro-adipogenic, were markedly decreased in the study, which ultimately inhibits adipocyte differentiation (Misawa et al, 2012). As in other studies, the administration of dietary aloe QDM complex in obese mice lowered the body weight and suppressed the expression of PPARγ which then averts the differentiation of adipocyte (Shin et al, 2012).

Beside testing *Aloe vera* on animals, *Aloe vera* has also been experimented on cell cultures. Commercial botanical products, including *Aloe vera*, were investigated fin the differentiation of 3T3-L1 adipocytes for effects on lipogenic activity (Babish et al, 2010). Surprisingly, the study showed the capability of *Aloe vera* in increasing lipid accumulation (Babish et al, 2010). Moreover, the resulting research is contradicts the study conducted by Anand et al. which examines the effect of Aloe-emodin, an anthraquinone compound isolated from *Aloe vera* leaf, on 3T3-L1 preadipocyte (Anand et al, 2010). Lipid accumulation was suppressed during the adipocyte differentiation using 3T3-L1 preadipocyte (Anand et al, 2010). Furthermore, adipocyte differentiation was also nearly blocked with 20 µM *Aloe vera* using human mesenchymal stem cells (Subash-babu and Alshatwi, 2012). Downregulation of PPARγ expression in the study is likely to cause an inhibitory effect in adipogenesis (Anand et al, 2010; Subash-babu and Alshatwi, 2012).

This study, however, did not show a statistically significant difference among treatments. The results also showed that the reduction in lipid absorbance value reduction was not in a consistent form in line with the increase of *Aloe vera* ethanol extract concentration. The 10 ppm group showed the lowest lipid absorbance value, whereas the 20 ppm group showed the highest lipid absorbance value among samples with *Aloe vera* treatments.The outcomes of previous studies and the current study showed some differences. These differences might occur due to several reasons. First, the *Aloe vera* used were obtained from different places. Second, the subjects used were different from one another. Some experiments assessed *Aloe vera*'s effect on mice while th others evaluated the effect on cell lines. In vivo and in vitro studies have a different characteristic, which might also differs the result. Third, the mice and cell lines were obtained from different areas and were not homogenous species. This might result in different outcomes with the addition of *Aloe vera* to different subjects.

This is the first experiment in Cell Culture Laboratory, Faculty of Medicine, Universitas Padjadjaran, which uses *Aloe vera*. Therefore, in conducting this study, we started by using a small amount of *Aloe vera* ethanol extracts concentration. This result might suggest that the concentrations used were not the optimum concentrations needed showing an inconsistent decline in the lipid accumulation. Further studies on protocol optimization in using *Aloe vera* ethanol extract on 3T3-L1 cell lines, and exploring the master regulatory gene of adipogenesis (PPARγ, C/EBP, SREBP) are strongly recommended for the forthcoming experiment.

CONCLUSION

In this study, the addition of *Aloe vera* ethanol extracts during 3T3-L1 adipocyte differentiation showed a decrease in lipid absorbance value even though statistically showed no significant differences ($p>0.05$). As this is the first study which uses *Aloe vera* ethanol extract on 3T3-L1 adipocyte differentiation, this study can serves as a guide for further studies in this field.

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Conflict of Interest: The authors declared no conflict of interest in this study.

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Accumulation of Cells in Sub-G1 Phase and Apoptosis Induction by a Bioactive Fraction from the Seaweed *Gelidiella acerosa*

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ABSTRACT

Marine algae or seaweeds serve as excellent resources of bioactive secondary metabolites with wide range of therapeutic applications and multiple biological activities. Despite the growing worth of algae as a source of pharmacological compounds, the biotechnological or anticancer application of marine algae are still under-exploited. Hence the present study is aimed at exploring the anti proliferative activity of the marine macroalga, *Gelediella acerosa* against HeLa,HepG2 and MCF-7 cancer cell lines and to identify the bioactive compound. The secondary metabolites of *G. acerosa* were extracted using methanol, the extract was purified by TLC. The bioactive fraction was selected through bioassay guided fractionation and the cytotoxic, apoptogenic potential of this fraction was analysed by different in vitro assays such as MTT assay, LDH assay, Trypan blue dye exclusion, DNA fragmentation, caspase activity and cell cycle analysis by flow cytometry. The characterization of the bioactive fraction was performed through GC MS analysis. The results of MTT and trypan blue assays indicated significant cytotoxicities to the GF7 treated HeLa, HepG2 and MCF-7 cancer cells and at the same time demonstrated non-toxicity to normal human lymphocytes at 50µg/mL concentration. The mechanism of action of this fraction on the cancer cells was observed as apoptosis induction as indicated by significantly elevated caspase activity, decreased cell counts, DNA fragmentation pattern and elevated LDH enzyme activities. Cell cycle analysis showed majority of cells accumulating in the sub-G1 phase that further confirmed apoptosis induction by the algal fraction. GC-MS analysis indicated the presence of hexadecanoic acid, previously documented for anticancer activity, that might be responsible for its bioactivity. It can be concluded that this algal bioactive fraction (GF7) has significant anti-cancer potential at low concentrations and shows promise for future in-vivo studies that might lead towards a safer anti cancer compound..

KEY WORDS: ANTI-CANCER, APOPTOSIS, CELL CYCLE, GELIDIELLA ACEROSA, SUB-G1.

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INTRODUCTION

Cancer is one of the major challenging diseases that leads to the death of several million people globally in an annual basis (Aggarwal et al., 2009). The fundamental characteristic of all types of cancers is uncontrolled cell division (Tay et al 2019). With most of the existing treatment modalities ineffective in completely curing the disease, there is a need to search for ideal anti-cancer agents from natural resources. It has been reported that uncontrolled symmetric cell division is the major factor that contributes to cancer. Hence it is quite natural for scientists to aim anticancer drugs that control cell cycle machineries and that induce apoptosis (Bai et al., 2017, Ashraf 2020).

Marine organisms with a wide range of bioactive secondary metabolites of pharmaceutical significance have the potential towards the development of anti cancer agents (Newman and Gordon, 2016). Marine algae comprise one of the rich sources of a wide range of secondary metabolites. The protective effect of edible seaweeds has been established against mammary, skin and intestinal carcinogenesis (Yuan and Walsh 2006). Marine algae have been used as food and also as traditional medicine in the eastern hemisphere (Kilinc et al., 2013). The red alga, *Gelidiella acerosa* is abundantly found in the coastal area of South India, especially in inter tidal region of Gulf of Mannar (Taskin et al., 2007; Bernecker et al., 2009). *G. acerosa* is mainly used for agar production and also has many phytochemical constituents having biological activities including anti cancer activities (Duraikannu et al., 2014; Begum et al., 2018).

Though anticancer activity was reported for *G. acerosa*, previous studies (both *in-vitro* and *in-vivo*) were carried out using crude extracts of this promising algal species. Hence we felt the necessity to purify and characterize the bioactive compounds from this red alga and aimed to study the mechanism of action of its bioactive components on cell cycle stages of cancer cell lines in the present study.

Figure 1: Macroscopic view of *Gelidiella acerosa*

MATERIAL AND METHODS

Preparation of algal extract and extraction of metabolites: Seaweed was collected from the Mandapam camp at Rameshwaram, Tamilnadu in India. The collected seaweed was identified as *Gelidiella acerosa* (Figure 1) and authenticated by Dr.Eswaran, Principle scientist at marine algal research station, Rameshwaram.*G. acerosa* was washed and dried for 7 days under the shade. The dried samplewas powdered with the help of amixer grinder and themetabolites were extracted in a soxhlet apparatus using methanol as the solvent. The extract was further concentrated in a rotary evaporator (IKA, Germany) at 40° C.

The methanol extract of G.acerosa was initially screened for cytotoxicity to cancer cell lines (HepG2, HeLa and MCF-7) by MTT cell viability assay.The methanol extract was further fractionated by TLC sheets using different solvent combinations.Through bioassay guided fractionation, the bioactive fraction was chosen for further cytotoxicity assays.

MTT Cell viability assay: MTT[3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide] assay is a colorimetric assay to determined the viability of the cells treated with the samples (Mosmann, 1983). The percentage viability of the cells was calculated as follows:

Percentage viability (%) = O.D 540 of treated / O.D 540 of control × 100.

Trypan Blue assay: Trypan blue dye exclusion test was carried out to determine the concentration of viable and dead cells in a cell suspension (Strober, 1997).Cells were treated with 50µg/ml of the sample for 48 hrs. The cell concentration was determined by counting the number of viable cells in the treated group with the help of a hemocytometer.

LDH cytotoxicity assay: The extent of damage in the treated cells was analysed by LDH cytotoxicity assay in the treated cells as per the protocol given in the kit manual (Weyermann et al., 2005).

Caspase -3,7 and 10 activities: The activity of caspase enzyme in the sample treated cancer cells was measured using CasPASE(tm) Apoptosis Colorimetric Assay kit (G Biosciences Ltd, USA),as per the instructions given in the manual of the kit manufacturer.

DNA fragmentation assay: DNA fragmentation is a semi quantitative method to analyse the fragmentation of DNA in the treated group in which the cells were harvested, DNA was extracted and loaded to 0.8% agarose gel for electrophoresis (Shidoji and Ogawa, 2004).

Flow cytometry analysis with Propidium Iodide staining: Flow cytometry analysis of the sample treated and untreated cancer cells was performed to analysethe distribution of DNA at different stages of the cell cycle. After 48 hrs of sample treatment, the cells were harvested

and the cell cycle was analysed as per the standard methodology (Pazarowski and Darzynkiewick, 2004).

GC-MS analysis: The GF7 fraction of *G. acerosa* was subjected to analysis through GC-MS method at the facility of Central Silk Technological Research Institute, Bangalore. The resulting mass spectral peaks of unknown compounds were analyzed and compared with the database of anti-cancer compounds so as to identify the bioactive component .

RESULTS AND DISCUSSION

Drug discovery from natural sources such as marine algae is an important area of recent research in cancer Biology. Many marine algae were found to produce structurally diverse secondary metabolites of therapeutic significance.In the current study, we mainly focussed toevaluate the anti cancer potential of the red alga *G. acerosa* towards in-vitro cancer cell lines. The methanol extract of *G.acerosa* was found to have anti proliferative activity to all the tested cancer cell lines at 24,48,72 and 96 h of treatment. The maximum inhibition of cell proliferation was observed at the higher concentration of 100µg/ml. The viability was 46.82% for HepG2, 40.38% for MCF-7 cells and 36.59% for HeLa cells after 96 h of treatment with 100µg/ml of the methanol extract of *G. acerosa* (Table 1).

Table 1. Effect of methanol extract of *G. acerosa* to various cancer cell lines at different concentrations.

Concentration (µg/ml)	Viability (%) HeLa				
	Control			Treated	
		24 h	48 h	72 h	96 h
1	100	100	83.31	71.65	49.97
10	100	100	82.61	62.77	68.51
50	100	100	76.63	53.40	37.72
100	100	98.1	66.20	51.82	36.59
	HepG2				
1	100	100	100	80.63	72.72
10	100	100	82.37	75.25	67.58
50	100	89.6	81.55	62.96	53.58
100	100	73.7	73.77	57.74	46.82
	MCF-7				
1	100	99.48	97.1	92.13	80.87
10	100	79.23	73.3	70.48	58.90
50	100	72.43	73.0	69.6	46.67
100	100	66.66	55.1	54.8	40.38

The methanol extract of *G. acerosa* was partially purified by thin layer chromatography using the solvent combination ofAcetonitrile: chloroform: dichloromethane: Toulene (1:2:2:1). Seven different fractions were observed under UV and visible lights. When each of the fractions were tested by MTT assay,the 7th fraction (GF7) demonstrated maximum cytotoxicity than the other fractions (results not shown). When the effect of GF7 was analysed on different cancer cell lines, an increase in the treatment concentration of GF7 (from 1 to 50 µg/ml) resulting in a decreased viability of cells was seen. The cell viability at 50µg/ml was 36.5 % for HeLa, 46.8% for HepG2 and 40.3% for MCF-7 cells after 96h treatment (Figure 2). The IC50 value of GF7 was calculated as 39 µg/ml for HeLa, 27µg/ml for HepG2 and 37µg/ml for MCF-7cells for 96h of treatment.

Figure 2: Percentage cell viability of the cancer cells treated with GF7 at different treatment periods of 24, 48, 72 and 96 hrs * represents significance at p<0.05,** represents significance at p<0.01.

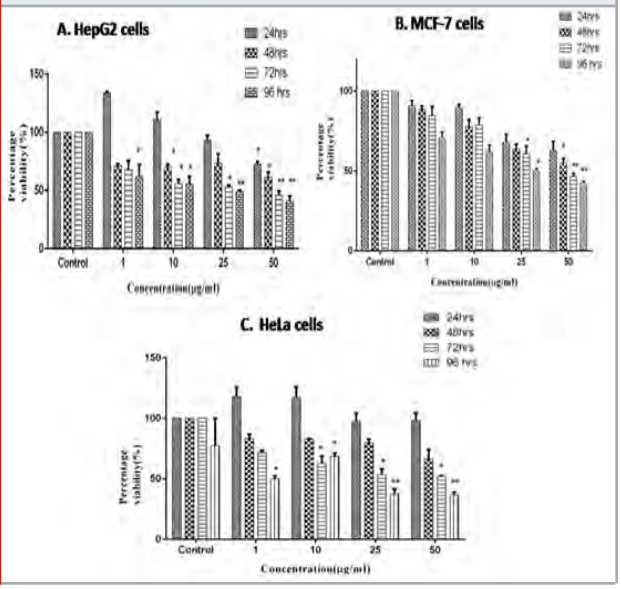


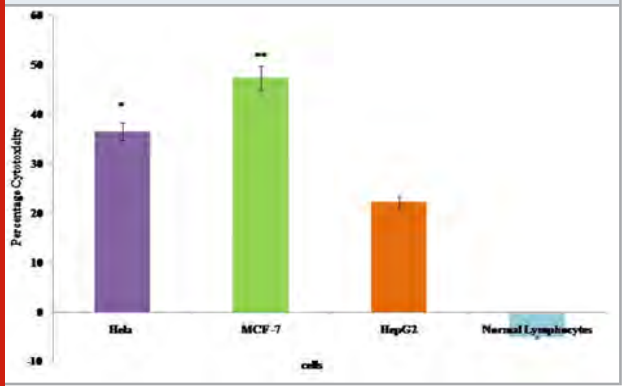
Table 2. Determination of cell count and cell viability by Trypan blue method.

Cell lines	Untreated Control		GF7 Treated	
	Viable Cell count (1x106 cells/ml)	Viability (%)	Viable Cell count (1x106 cells/ml)	Viability (%)
HeLa	8.56	99.76	3.29	38.79
MCF-7	9.93	96.59	3.62	35.01
HepG2	9.81	98.29	3.57	35.87
Normal Lymphocytes	1.51	98.69	0.97	97.53

In a previous report (Lakmal et al., 2014), where the anticancer activity of six different seaweeds were analysed, *G. acerosa* had moderate inhibition against HL-60 cells and had no cytotoxicity against mouse melanoma (B16F10) and human lung carcinoma (A549) cells. As compared to this report, the present study

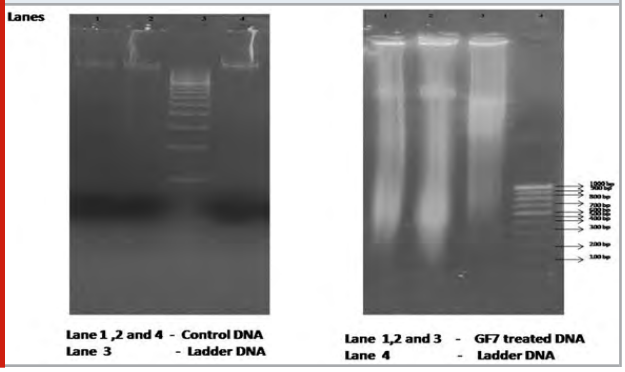
shows promising anti-cancer activity of *G. acerosa*. When the effect of GF7 on HepG2, HeLa and MCF-7 cell concentration was examined by trypan blue assay, it was clear that all the cell lines treated with the bioactive fraction GF7 had a decreased viable cell count as compared to the control cells (Table 2). There were $3.29x10^6$ cells/ml in HeLa, $3.2X10^6$ cells/ml in MCF-7 and $3.57x10^6$ cells/ml in HepG2 cells after 48h of treatment with the viability ranging between 35.0-38.79%. No cytotoxicity was observed to the treated normal lymphocytes.

Figure 3: LDH cytotoxicity of the cancer cells treated with GF7.



The cytotoxic effect of the bioactive fraction GF7 to the cancer cells was assessed by LDH assay. Changes in the membrane integrity or membrane damage cause the release of LDH enzyme into extra cellular media. The cytotoxicity caused by GF7 fraction was 36.47% on HeLa, 47.38% on MCF-7 and 22.25% on HepG2 cells as compared to the control cells. No cytotoxic effect was observed tonormal human lymphocytes by GF7 treatment (Figure 3).

Figure 4: DNA fragmentation analysis of cells treated with fraction GF7



The hallmark of apoptosis is the degradation of DNAinto fragments by endogenous DNAses. The mechanism of cell death caused by GF7 is determined by DNA fragmentation analysis (Saraste and Pulkki, 2000; Elmore, 2007).Based on the results of this analysis, we could see shearing of DNA in the treated cancer cells as compared to the control cells, where the DNA was intact and a single band was visible (Figure 4).

Caspases are a family of aspartate-specific cysteine proteases and detection of activation of caspase activity is a valid method for assessing apoptosis (Chang and Yang, 2000).In our study, cells treated with GF7 had significantly higher caspase activity (Figure 5). The percentage increase in caspase activity of HepG2, HeLa and MCF-7 cells were 67.20%,16.02% and 7.9% respectively. When cell cycle stages were analysed by flow cytometry, we observed that GF7 treated cancer cells had increased number of dead cells which accumulated in the sub G1 phase of the cell cycle (Figure 6).Sub G1 indicates apoptotic cells. The concentration of sub-G1 phase was 81.7% in HepG2, 68.4% in MCF-7and 80.4% in HeLa cells.

Figure 5: Caspase activity in cancer cells treated with 50µg/ml of fraction GF7.* represent significant difference ($p<0.05$) between treated and untreated cells

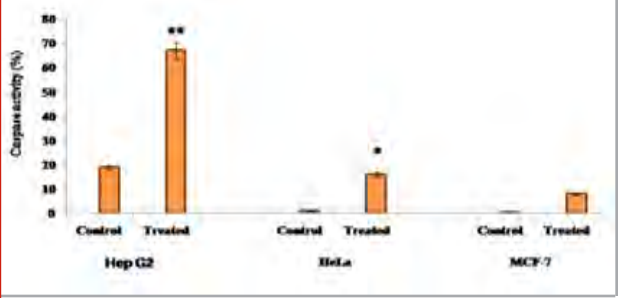
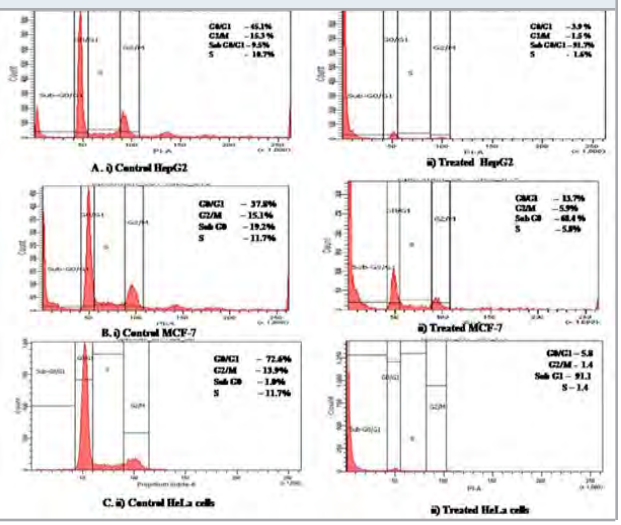


Figure 6: Flow cytometry analysis of cell cycle stages by propidium iodide staining in cancer cells untreated and treated with GF7.

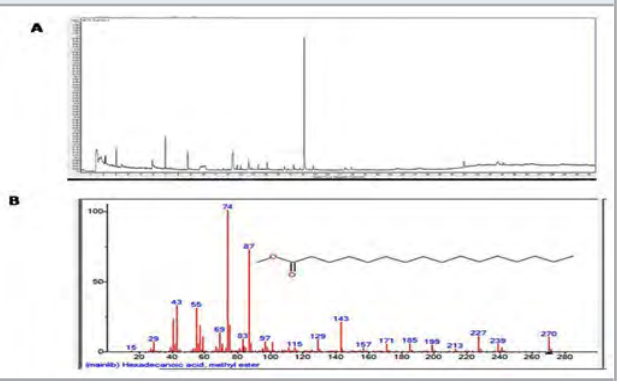


As the bioactive fraction GF7 from *G. acerosa* showed significant apoptogenic potential towards cancer cells, this fraction was subjected to GC-MS method for characterization. From the results, we found hexadecanoic acid in the major peak at RT 18.3 min (Figure 7).

Apoptosis induction is an important strategy to control and manage cancer. Cell cycle arrest at key points such as G1, S, G2 and G2/M drives the cells through apoptosis.

Many synthetic or natural anticancer compounds lead to cell cycle arrest and apoptosis as reported by many researchers (Alabsi et al., 2013; Wafa et al., 2020). In the current study, we could establish the anticancer potential of the methanol extract of the marine alga *G. acerosa*, to cervical, breast and liver cancer cell lines at lower concentrations of 25 and 50µg/ml when compared to earlier reports (Murugan and Iyer, 2013; Lakmal et al., 2014; Duraikannu et al., 2014; Begum et al., 2018).

Figure 7: GC-MS analysis of fraction GF7. A. Gas Chromatogram and B. mass spectrum of fraction GF7 along with the Structure of Hexadecanoic acid



In a previous study, the methanol extract of *G. acerosa* has shown cytotoxicity to HL60 cancer cell line at higher concentrations of 100 and 200µg/ml, where the IC_{50} value was reported as 104.4 µg/ml (Lakmal et al., 2014). In another much recent study the IC_{50} value of *G. acerosa* against lung cancer cells was reported as 1.5 mg/ml (Begum et al., 2018). As compared to these earlier study reports, the crude methanol extract of *G. acerosa* in our present study we got higher cytotoxicity to all the tested cancer cell lines with IC_{50} values <50 µg/ml for 96h of treatment duration and after purification by TLC, the bioactive GF7 fraction had much greater cytotoxicity with IC_{50} values with IC_{50} values further decreasing, with a range of 27µg/ml -39 µg/ml towards HeLa, MCF-7 & HepG2 cancer cells.

The bioactive compounds which are present in crude extracts at very low concentrations get concentrated during the purification process and that could be the reason for higher specificity and low IC50 value of *G. Acerosa* purified fraction in the current study. There are reports from many other red algae having cytotoxicity to human cancer cell lines (Harada and Kamei, 1997; El, Baroty et al., 2007). In a previous study, *G. acerosa* was reported to reduce cancer cell growth and tumor weight in mice at 200mg/kg body weight (Duraikannu et al., 2014). In another study *G. acerosa* crude extract was reported as to have cytotoxicity to cancer cells and they also reported that the presence of polyphenols and flavonoids in the ethyl acetate extract of *G. acerosa* (GAE) being is the reason for inhibition of lung cancer cell proliferation at 1.5 mg/mL (Begum et al., 2018).

On the contrary, in our study we report the presence of Hexadecanoic acid in the bioactive fraction of *G. acerosa* as per the results of GC-MS analysis. Hexadecanoic acid is a well documented for anticancer potential and is reported from some other marine algae also (Parveen and Nadumane 2020). To the best of our knowledge, we are reporting its presence for the first time from *G. acerosa*. We also found that the basis for the anticancer mechanism of *G. acerosa* is cell cycle arrest, accumulation of cells in subG1 phase leading to the apoptosis of treated cancer cells. In our study apoptosis induction was clearly seen in DNA fragmentation, increased caspase activity, cell cycle stages, LDH cytotoxicity and highly decreased cancer cell counts due to *G. acerosa* treatment. Though there are previous studies reporting the in-vitro and in-vivo cytotoxicity of the crude extracts of *G. acerosa*, no attempts were made to purify the active component involved in the reported activity.

To the best of our knowledge,this study is the first attempt to analyse the mechanism of anti cancer action of TLC purified fraction of *G. acerosa* and characterizing the bioactive compound. Through the results of the present study, we report the presence of hexadecanoic acid in the bioactive fraction and assume it to be the reason for the observed anti cancer activity of *G. acerosa*. Fatty acids comprise one of the predominant phytochemical constituents in marine macroalgae and reported to have many biological activities (Hema et al., 2011). Palmitic acid (n-hexadecanoic acid) is documented to have anticancer potential with apoptosis inducing ability in human cancer cell lines (Yoo et al., 2007; Mericli et al., 2017; Parveen and Nadumane 2020). Our results too are validating these earlier reports.

CONCLUSION

Through the present work, it can be concluded that *G. acerosa* has cytotoxic activity against human cancer cell lines along with no toxicity to normal human lymphocytes. Presence of Hexadecanoic acid in the bioactive fraction GF7, might be the reason for the anti cancer activity of *G. acerosa*. Further studies need to be carried out for the development of an efficient drug against cancer from this promising Marine red alga.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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A Review on Brain-Computer Interface Spellers: P300 Speller

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ABSTRACT

People with Locked-In Syndrome (LIS) have neuromuscular disabilities. Due to these lost and other situations, the concept of Brain-Computer Interface (BCI) established which translates brain wave activity to meaningful commands and helps to obtain direct connection between human brain activity and the world. It is the process of digitizing brain signals to perform specific commands on an external device. One of the BCI popular applications is the BCI speller especially the P300 speller that provides an alternative communication way for people with neuromuscular disabilities. It contains a screen of characters represented as a matrix, by which patients can select a character from it based on an Event-Related Potential (ERP) response that appears in their brain waves activity as a positive-going wave within about 300 milliseconds after selecting a desired character i.e. target, during the spelling process. In this review, we attempt to illustrate different versions of P300 spellers and describe the different paradigms of P300 speller by classifying them according to the spelling medium, the number of layers, and the flashing patterns. These paradigms including Audio P300 Speller Paradigm, Visual P300 Speller Paradigm, Visual Uni-modal P300 Speller Paradigm, Visual Bi-modal P300 Speller Paradigm, Visual Multimodal P300 Speller Paradigm, and Audio-visual P300 Speller Paradigm. We conclude this paper with some open issues in different areas, which are brain signals records, flashing patterns, paradigms, spelling duration, and BCI software. Therefore, according to the variety of P300 speller paradigms, many areas can be developed to enhance spelling performance and improve its utilization quality.

KEY WORDS: BRAIN-COMPUTER INTERFACE (BCI), P300 SPELLER, LOCKED-IN SYNDROME (LIS), OPEN ISSUES.

INTRODUCTION

Communication is the most important and simplest way to express feelings and needs in a person daily life. As normal people, it can be done through several ways, such as speaking, writing, etc. People with severe motor disabilities, e.g. Locked in Syndrome (LIS) and Amyotrophic Lateral Sclerosis (ALS), have limited capability of communication or transfer information to others Vidal (2020). There should be an alternative communication way to help in improving patients' life quality. As a result of this problem, the concept of Brain-Computer Interface (BCI) innovated which translate brain signals to perform specific commands. Many researches have begun to search, improve this domain, develop algorithms and innovate some applications based on this technology. In order to provide easy ways of communications with people who suffer from severe neuromuscular disabilities.

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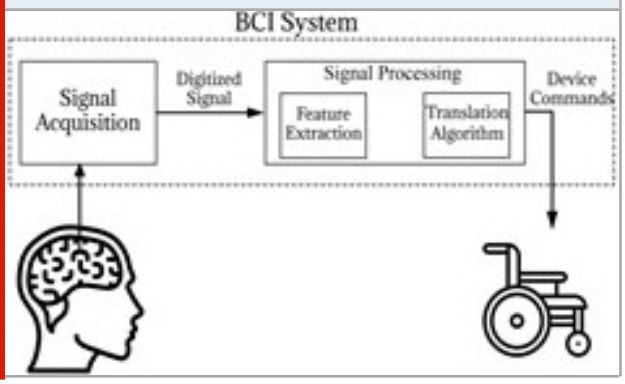
In the early 1920s, scientists explored that human brain produces electric currents and they were able to measure it by electrodes, which Electroencephalogram (EEG) considers as one of the tools that invented in 1929s which resulted a huge impact in neuroscience Nam et al. (2018). Based on EEG responses, human brain waves can be translated to perform a specific command Naufel & Klein (2020) and Ratcliffe et al. (2020). BCI applications varies based on their uses, one of the popular BCI applications is BCI speller which will be discussed during this review. BCI spellers differ based on the medium implemented in, flashing patterns used, as well as speller Graphical User Interface (GUI).

This review aims to provide some research areas on enhancing P300 speller by illustrating some of the existing versions of it and discussing the related open issues. The review consists of a background on BCI technology, its classes, types, and platforms in the second section, a detailed explanation of the different P300 spellers in section 3, the fourth section discuss some of P300 speller-based open issues, and a concluding statement.

Brain Computer Interface (BCI) definition and classes: BCI helps to get direct commu-nication between brain activity and an external device to perform an action Schalk et al. (2004). It is the process of digitizing brain signals to perform certain commands on an external device. Brain waves are the main component in the BCI system. The system starts with acquiring brain signals, processing these signals then execute a specific action. Figure 1 illustrates the structure of any BCI system in which brain signals are detected by the electrodes on human scalp, skull or within human brain, and are processed to extract desired features which consider as user's intents. The features are translated to command and operate a device, e.g. a wheelchair, Khairullah et al. (2020).

Figure 1: Basic Structure of any BCI System.



The main aim of any BCI system is to engage users with severe motor disabilities with the society, make their daily life communications easier, and grant smart living space for them Lin & Hsieh (2016). BCI technology is also applied in education for normal people (NeuroSky, n.d.), entertainment Andujar et al. (2019) and Rosca & Leba (2019), and Three-Dimensions (3D) virtual environments Andujar et al. (2019) and Vishwakarma (2020). A BCI system is classified into two classes:

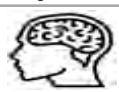
Dependent BCI system: In dependent BCI systems, there is a dependency on the muscular and nervous system in brain signals translating process such as, gaze direction or muscle movement. A simple case of a dependent BCI system, patient spells a word using a matrix with the help of eye tracker device, by focusing the gaze direction on the desired character. When that character flashes a Visual Evoked Potential (VEP) is recorded from the visual cortex in the patient scalp.

Independent BCI system: On the other hand, the independent BCI system relies on patient's attention i.e. intent, not on the muscular system. For example, using a matrix that flashes to spell a word. When the desired character flashes, a specific change in patient's brain signals is detected known as P300 Event Related Potential (ERP). This class depends only on user intent while reading brain signals.

MATERIAL AND METHODS

There are three types of BCI systems: invasive, semi-invasive, and non-invasive Gonfalonieri (2018). Table 1 presents an overview of these types. Invasive and semi-invasive BCI systems are mostly used to assist patients with severe neuromuscular disorder diseases such as Parkinson, to live a normal life Adama & Bogdan (2016).

Table 1. The Main BCI System Types.

Features	Invasive	Semi-invasive	Non-invasive
Electrode placement	Inside the brain	Inside the skull	Above the scalp
Risk rate	Risky	Risky	Harmless
Cost	Expensive	Expensive	Inexpensive
Described image			

BCI platforms: During the last four decades, researchers have been giving their attention to BCI systems and platforms Velasco-Álvarez et al. (2019). As a result, they produced several software tools for BCI development and analysis that scientists and researchers can use in their experimental studies. Samples of the software tools will be briefly described in this subsection.

BCI2000: a free open source BCI software built in C++ Schalk et al. (2004). It supports different data acquisition devices, signal processing algorithms, and experimental paradigms. It also allows paradigm customization. The software can be discovered and installed from https://www.bci2000.org.

OpenViBE: a free GUI open source BCI software Arrouët

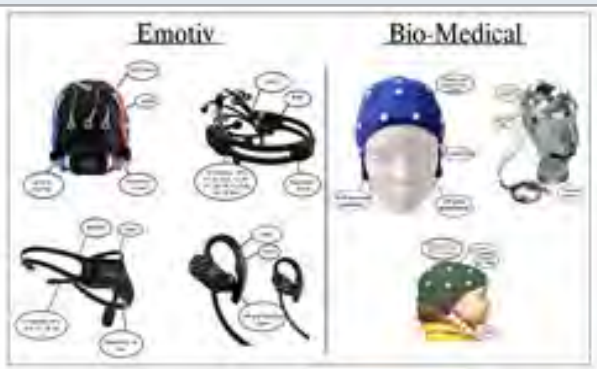
et al. (2005). It consists of modules their integration develops a full BCI system.

BCILAB: a MATLAB based open source software Kothe & Makeig (2013). This tool used in advancement of the BCI technology studies.

UMA-BCI Speller: a BCI2000 based software Velasco-Álvarez et al. (2019). The aim of producing this software is to provide a GUI MATLAB speller customization tool for non-technical researchers.

Electroencephalogram (EEG): EEG is a way of recording brain waves activity by placing electrodes on the patient scalp Paz & West (2014). Human brain consists of 6 lobes: frontal, parietal, temporal, occipital, cerebellum, and limbic Brain Regions & Functions (2010). Each lobe is responsible for performing many functions. Due to the variety of brain lobes and their functions, there are various electrode channels distributed around human scalp. Electrode layout differ based on the system it follows, 10-20 and 10-10 distribution systems are the most popular followed systems Morley et al. (2016) and Schoenberg & Scott (2011). Electrode channels cover brain lobes: frontal (F), temporal (T), central (C), parietal (P), and occipital (O). These channels are equally divided into three sections: right, left and center. Channels are named based on the brain lobe and location e.g. F3, F refers to frontal region located in the left side of the scalp as 3 is an odd number. All channels in the left section ends with odd numbers, whereas channels in the right section ends with an even number and centralized channels ends with letter z. Furthermore, there are many EEG products in the market used to record brain signals activity. Figure 2 presents samples of EEG products with some details Bio-medical (2019) and Emotiv (2019).

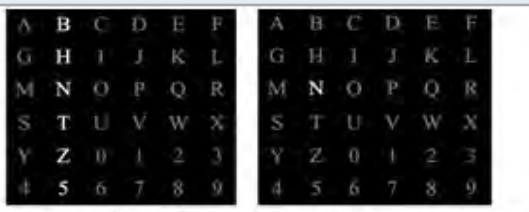
Figure 2: Some of EEG Devices by Emotiv And Bio-Medical Companies.



BCI spellers (P300 speller): BCI spellers are one of the BCI applications that have been considered by several researchers in both dependent and independent BCI fields Farwell & Donchin (1988). P300 speller is an independent BCI speller. It contains a screen of characters i.e. matrix, by which patients can select a character from it based on an ERP response that appears in their brain waves activity as a positive-going wave within about 300 milliseconds (MS) after selecting a desired character i.e. target, during the spelling process. P300 speller contains three main components: an EEG record device which records brain waves activity, a matrix of characters displayed on a screen, and a BCI software responsible for the processing and returning a feedback. After placing EEG device on patient's head, the screen will flash randomly by Single Character (SC), Row-Column (RC), or Region-Based (RB), covering all characters. After 300ms in which the desired character or region flashes, a P300 appears in brain records, the repetition of this appearance result on choosing that character and present it in the feedback bar on the screen, and the process continues till the desired word is displayed in the feedback bar. P300 speller performance depends on two factors: accuracy i.e. the percentage of the correct choices over all selections, and Information Transfer Rate (ITR) i.e. the amount of information transferred by system output measured in bits/trail or flash Lu et al. (2019) and Rezeika et al. (2018).

P300 speller matrix can be any size based on the needs. It also could be unimodal i.e. single layer Fernández-Rodríguez et al. (2019), Kabbara et al. (2015), Li et al. (2019), Qu et al. (2018), Ron-Angevin et al. (2019), and Speier et al. (2018), bi-modal i.e. two layers Pan et al. (2013) and Utsumi et al. (2018), or multi-modal paradigm Warren & Randolph (2019). As well as the flashes could be RB flashes Pan et al. (2013), RC flashes Guger et al. (2009), or SC flashes Guger et al. (2009). P300 speller could be performed visually using flashes Fernández Rodríguez et al. (2019), Kabbara et al. (2015) Li et al. (2019), Pan et al. (2013), Qu et al. (2018), Ron-Angevin et al. (2019), Salvaris & Sepulveda (2009), Speier et al. (2018), Utsumi et al. (2018), Warren & Randolph(2019), auditorily Hill et al. (2004), or audio-visual Lu et al. (2019) and Oralhan (2019). This review attempts to discuss the different versions of the visual P300 speller. Spellers comes in different languages rather than English, in order to cover as many patients as possible, e.g. Arabic Kabbara et al. (2015), Korean Rezeika et al. (2018), and Japanese Utsumi et al. (2018).

Figure 3: RC and SC Flashing Patterns in P300 Speller.



(a) Row-Column (RC) flashing. (b) Row-Column (RC) flashing.

RESULTS AND DISCUSSION

P300 speller flashing patterns: P300 speller has many diverse paradigms based on the flashing patterns Guger et al. (2009). Figure 3 illustrates two different flashing patterns RC, and SC. In RC pattern, multiple rows or/ and columns flashes randomly. On the other hand, in SC pattern, one random character is flashed at a time.

Guger et al. (2009) conducted a comparison between the performance of RC and SC P300 spellers with 100 participants. Participants, i.e. subjects, were free to choose the speller type. 81 participants experienced RC speller while 19 participants tested both versions. Subjects were asked to spell predefined words and answering some questions about education, sex, and a question related to their work. The result of this comparison showed that higher accuracy rate recorded in RC speller presented in 72.8%, whereas 55.3% was the recorded accuracy of SC flashing pattern. Moreover, RC pattern resulted a faster spelling process, a character flashes in RC each 28.8s, while in SC it flashes each 54s. Thus, the RC pattern is two time faster than SC pattern. The following subsections will describe the different paradigms of P300 speller by classifying them according to the spelling medium, the number of layers, and the flashing patterns.

Audio P300 Speller Paradigm: An auditory paradigm was developed to offer binary selection for LIS patients Hill et al. (2004). This paradigm relies on user attention to the spoken choice, the choice was heard from either left or right ear. Users hear binary choices; each choice is spoken near a specific side randomly. After number of trails and with the interpreting of EEG records, the desired selection will be identified. Auditory paradigms majorly impact in increasing blind LIS patients' involvement in communication. However, many artifacts can affect patients' selection. Thus, complex signals classification is necessary.

Visual P300 Speller Paradigm: The first P300 speller was introduced which is a visual BCI speller paradigm Farwell & Donchin (1988). This paradigm consists of 6*6 matrix that contains the 26 alphabet letters and 1-word controlling commands, presented in a screen to LIS patients as shown in Figure 4. A number of flashes by row or column used to detect the desired character, 12 flashes required to cover all cells, i.e. characters. The spelling process begins with recording patient's brain waves activity using an EEG device while the matrix flashes. When the desired character flashes a P300 will be detected in the patient's brain wave records. The selected character will be shown in the screen as a feedback.

Figure 4: The first P300 speller paradigm.

Authors experimented their studies on four healthy volunteers in two sessions. In the first session they were asked to spell the word "BRAIN" and then choose the TALK command, to assess practicality of the technology and understand its procedures. All volunteers achieved the first session successfully with 30 trails, i.e. 30 RC flashes for each character, concluded with 6 symbols selection in 180 trails (each trail present for 100 ms). The second session aimed to compare between four classification algorithms to assess their efficiency, the compared algorithms were: Stepwise Discriminant Analysis (SWDA), peak picking, area, and covariance. SWDA showed as the fastest algorithm. The maximum accuracy achieved is 95% at speed 12 bit/min, i.e. 2.3 characters/minute, which could be a slow spelling process. Based on the studies we have read about BCI spellers, we noticed a huge involvement in the field of enhancing P300 spellers. Researchers are trying to make improvements in the signal's classification process, speller paradigm style, and results accuracy.

Visual P300 speller paradigm was originally a single-layer, i.e. Fernández-Rodríguez et al. (2019), Kabbara et al. (2015), Li et al. (2019), Qu et al. (2018), Ron-Angevin et al. (2019), and Speier et al. (2018), then researchers developed efficient paradigm consisting of two-layers, i.e. bi-modal paradigm Pan et al. (2013) and Utsumi et al. (2018). Bi-modal paradigm generates higher spelling accuracy and effective spelling process. Years later, researchers used multi-layers paradigm, i.e. multi-modal, to cover special communications, such as using P300 spellers to communicate in social media application e.g. Facebook Warren & Randolph (2019).

Visual Uni-modal P300 Speller Paradigm: Visual unimodal P300 speller paradigm consists of single layer contains characters Fernández-Rodríguez et al. (2019), Kabbara et al. (2015), Li et al. (2019), Qu et al. (2018), Ron-Angevin et al. (2019), Salvaris & Sepulveda (2009), and Speier et al. (2018). The flashing patterns on this paradigm differ from use to another, it could be RC or SC flashes Guger et al. (2009).

Salvaris & Sepulveda (2009) experienced different visual layouts with eight volunteers to indicate the preferable layout for users. White background, black background, small character size, large character size, and other visual styles are used with RC flashing pattern. One participant preferred the white background and another participant preferred the small character size. However, the remaining six participants did not prefer any specific layout. Thus, we can not specify a specific layout to be the best layout. But we believe that black background increases speller usability as user attention is not as distracted as in bright background colors. Moreover, Ron-Angevin et al. (2019) recorded highest usability with the medium character size.

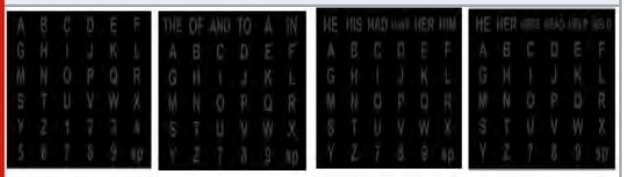
Speller size has a significant impact on the usability of P300 speller Ron-Angevin et al. (2019). Researchers compared between three different speller sizes in order to study the effect of speller size on the usability of P300 speller in terms of efficiency, effectiveness and satis-faction. Small, medium, and large speller sizes

are used. Twelve volunteers experienced the different speller sizes in six sessions each, while applying RC flashes. Each speller size is experienced twice for each attentional condition, overt and covert. Small and large speller sizes obtained poor results under covert attention, as the distances between characters are too small and too large respectively. Thus, small and large speller sizes considered as complex, tiring, and uncomfortable to its users. Furthermore, the medium speller size was the recommended size, since its results showed it was the most effective, efficient, and satisfied speller size in term of usability.

Kabbara et al. (2015) generated the first Arabic speller, by using a 6*5 matrix represents Arabic letters with RC flashes as illustrated in Figure 5. Eleven healthy participants participated in four sessions. They were asked to spell separated letters during the first and second sessions, while spelling words in the third and fourth sessions. Authors had proved that Support Vector Machine (SVM) classifier is the best feature classifier, after comparing it with other three feature extraction and classification algorithms.

Figure 5: Arabic visual P300 speller.

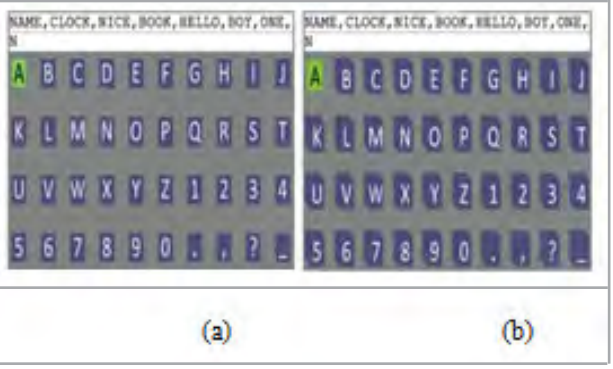
Figure 6. Predictive P300 speller. (a) Standard matrix, (b) Suggestion of 6 words of the most likely to choose, while numbers from 1 to 6 are removed, (c) After entering "H" letter, and (d) After entering the second character "E".



Speier et al. (2018) improved a language suggestion paradigm by combining a predictive method with a filtering method to enhance the performance of suggestion paradigms. Their goal was to use a filtering method to extend the signal classification for P300 speller to enhance the predictive method results. Twelve healthy volunteers experienced in this experiment and RC flashes pattern used. During spelling process, suggestions were shown in top of the matrix. Meanwhile, numbers from 1 to 6 disappeared as shown in Figure 6.

This study resulted in increasing the typing rate by 15.5% Speier et al. (2018). Due to this paradigm, subjects were able to spell a full word once. In contract, there are many limitations and challenges facing language modelling, e.g. words that are not included in vocabulary will not be supported. Qu et al. (2018) proposed a 3D P300 speller paradigm and compared it with the traditional Two-Dimensions (2D) speller. Both paradigms have the same sizes, SC flashing pattern, and layout in which non-flashed character has a blue background, and whenever a character flashes its background color turns into green as presented in Figure 7. However, 3D paradigm symbols in cubes, when a cube flashes a 3D motion will be shown. Twelve healthy participants joined in this study; they were asked to wear 3D glasses while experimenting the spelling process with the 3D paradigm. The 3D visual P300 speller recorded improvements on the spelling performance, higher accuracy, and lower user workload than the traditional 2D visual P300 speller.

Figure 7: 2D versus 3D visual P300 speller paradigms.



Although researchers had worked on enhancing the performance of visual P300 speller, it is still inadequate Li et al. (2019). As a consequence, authors developed a new colored smiley icon-faces speller paradigm that relies on user mental effort. A 6*6 matrix contains alphabets and numbers from 0 to 9 is used. SC flashing pattern was followed, but in an alternative flashing appearance, whenever the character flashes, the character will be switched to a colored smiley icon-face as described in Figure 8. Although this paradigm increased the spelling accuracy it increased the spelling process period.

Figure 8: Colored smiley icon-faces P300 speller paradigm.

Fernández-Rodríguez et al. (2019) focused on stimuli kinds that may enhance the performance of BCI speller. The intention of this paper was to compare the

Figure 9: Picture based P300 speller paradigm.

performance of the traditional character-based paradigm with picture-based paradigm as shown in Figure 9.

Fernández-Rodríguez et al. (2019) involved twenty-three participants. Results showed that their paradigm had higher performance than the traditional version. Although picture-based paradigm produces faster spelling process, it has limited spelling choices. It is important to mention that picture-based matrix may not be suitable for all patients due to possibility for the appearance of improper pictures such as violence, or nudity.

Figure 10: SC uni-modal P300 speller.

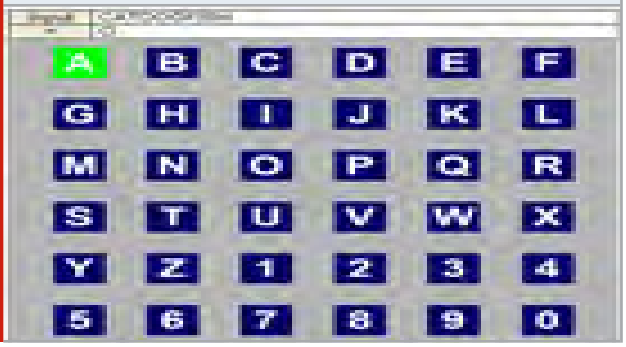
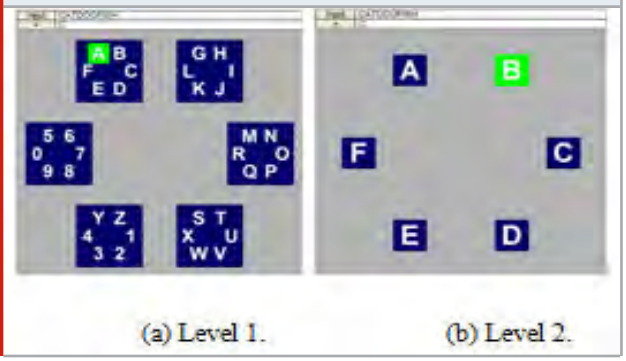


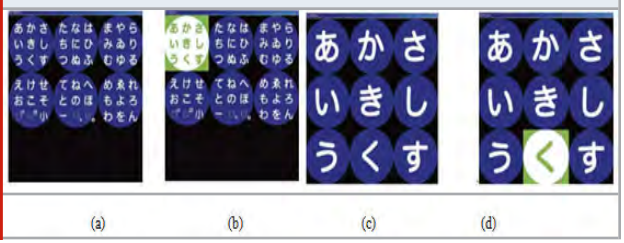
Figure 11: RB bi-modal P300 speller.



Visual Bi-modal P300 Speller Paradigm: Many improvements achieved in speller GUI. Pan et al. (2013) focused on SC paradigm which has been described earlier and RB paradigm. RB was conducted with the arrangement of six characters into six groups in the first level as shown in Figure 10 and 11. Seven healthy volunteers participated in this experiment. With twelve random flashes in first round, each region randomly flipped for 75 ms, users were asked to choose a region that contains the desired character from level 1, after that the second level appears which contains six characters, and each character will be flashed randomly. In order to compare between the two versions, the same conditions were applied.

Subjects were asked to spell three given words as their first task, while applying 10 trails per character Pan et al. (2013). The second task was testing the performance accuracy in many repeats. Each session contains specific number of runs. In each run, subjects were asked to pay attention to some letters to build specific words. According to the first task, the recorded average accuracy of SC was 84.26% and for RB was 88.57%. For the repeated tasks, RB achieved 90% accuracy rate in 4 repeats while SC required 6 repeats to reach high accuracy. The research resulted that RB speller has better performance than SC according to the achieved accuracy rate. Duchenne Muscular Dystrophy (DMD) patients suffer from muscle weakness, although they can sometime communicate in many ways, muscle weakness makes it hard for them to use these communication methods continuously Utsumi et al. (2018). Utsumi et al (2018) thought that a BCI communication channel will be suitable for them as it does not require any muscle effort. They developed an RB two layers i.e. bi-modal, visual P300 speller paradigm illustrated in Figure 12.

Figure 12: Japanese RB bi-modal visual P300 speller paradigm. In (a) level 1 presents before flashing, while (b) shows level 1 with flashes, then (c) is selected as level 2, and the target selection presents in (d).



The first level contains 6 regions with 9 characters each Utsumi et al. (2018). Each region flashes randomly, whenever the desired region is detected, the second level will appear with 9 characters. Each character flashes randomly, then the desired character will be selected. Eight DMD patients participated in this experiment and reported accuracy rate of 79.8%. According to the reported accuracy rate, P300 speller may be beneficial for DMD patients.

Visual Multimodal P300 Speller Paradigm: A multimodal P300 speller paradigm was developed to introduce a new communication way with Facebook social media platform by relying on P300 signals only, without any muscle movement required Warren & Randolph

(2019). Figure 13 presents the paradigm as they called it "Facebrain", through this paradigm users are able to do many actions, such as searching, posting posts, chatting with others, and viewing profiles in Facebook.

Figure 13: Visual multimodal P300 speller: Facebrain.

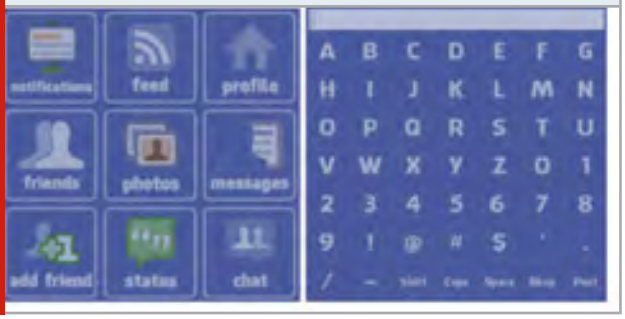


Figure 14: Audio-visual RB P300 speller paradigm sample 1.

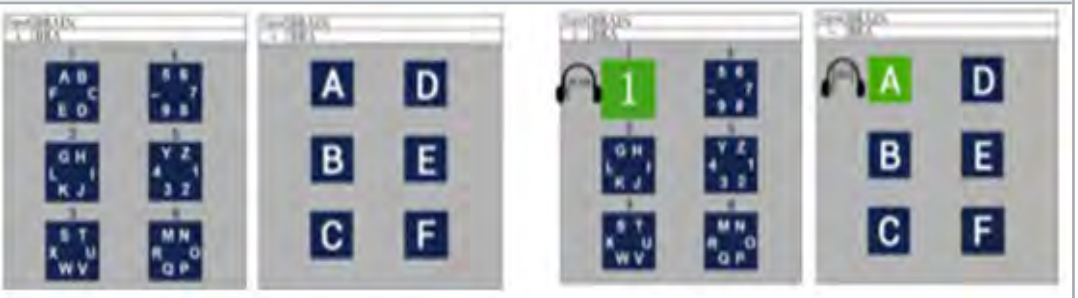
(a) Level 1. (b) Level 2.

Audio-visual P300 Speller Paradigm: The concept of developing visual P300 speller supported by sound generates a new P300 speller paradigm named as audio-visual P300 spellers Lu et al. (2019) and Oralhan (2019). Oralhan (2019) generated an audio-visual paradigm, and its performance was compared with audio-only paradigm and visual-only paradigm. The structure of the developed audio-visual paradigm is presented in Figure 14. The experiment reported a 90.13% accuracy with audio-visual paradigm, while 78.06% and 54.08% accuracy rates of visual-only and audio-only paradigms respectively.

Lu et al. (2019) continued the development of audio-visual spellers. Authors performed a comparison between visual and audio-visual RB P300 speller paradigm. Although the audio-visual paradigm recorded higher accuracy rate, a significant latency rate was recorded due to the focusing on increasing the accuracy. Figure 15 shows the paradigm they used. A summary of the discussed different paradigms of P300 speller is presented in Table 2.

Open issues on P300 speller: Regarding to the developments mentioned in previous studies there are some research areas that researchers could focus on as they may have a major effect in the performance of P300 speller. P300 speller performance could be affected by many factors, such as brain signals records, flashing patterns, paradigms, spelling spent time, and BCI software.

Figure 15: Audio-visual RB P300 speller paradigm sample 2.



(a) Initial state of level 1. (b) Initial state of level 2. (c) Flashing state of level 1. (d) Flashing state of level 2.

Figure 16: P300 speller-based open issues.

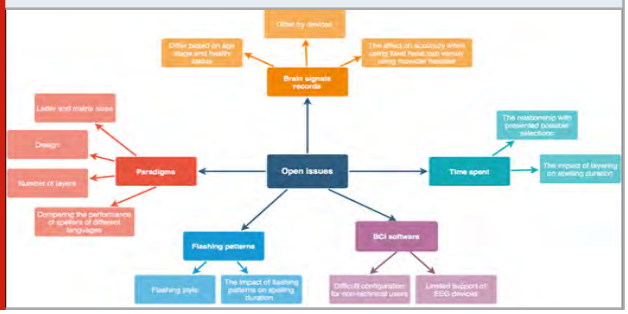


Figure 16 illustrates P300 speller-based open issues. Participants with different age stages and health status could change the brain signals records.

In addition, the various EEG devices could modify the acquiring accuracy. Moreover, while many researchers compared between the performance of RC and SC flashing patterns, applying other flashing styles may influence the spelling process. More than that, studying the impact of flashing patterns on spelling duration would be useful in reducing the spelling spent time. Furthermore, modifications on P300 speller paradigm could enhance its performance, e.g. propose new designs, change

Table 2. Summary of different P300 speller paradigms.

Paradigm Type	Number of Choices	Number of Layers	Flashing Pattern	Number of subjects	Reference
Auditory	Two	One	-	15 healthy	Hill et al. (2004)
Visual	Unlimited	One	RC	4 healthy	Farwell & Donchin (1988)
Visual	Unlimited	One	RC	8 healthy	Salvaris & Sepulveda (2009)
Visual	Unlimited	One	RC	12 healthy	Ron-Angevin et al. (2019)
Visual	Unlimited	One	RC	11 healthy	Kabbara et al. (2015)
Visual	Unlimited	One	RC	12 healthy	Speier et al. (2018)
Visual	Unlimited	One	SC	12 healthy	Qu et al. (2018)
Visual	Unlimited	One	SC	27 healthy	Li et al. (2019)
Visual	Unlimited	One	SC	23 healthy	Fernández-Rodríguez et al. (2019)
Visual	Unlimited	Two	SC, RB	7 healthy	Pan et al. (2013)
Visual	Unlimited	Two	RB	8 patients	Utsumi et al. (2018)
Visual	Unlimited	Multilayers	RC	In lab only	Warren & Randolph (2019)
Audio-Visual	Unlimited	Two	RB	7 healthy	Oralhan (2019)
Audio-Visual	Unlimited	Two	RB	18 healthy	Lu et al. (2019)

symbols size, and apply different number of layers. Comparing between spellers of various languages would affect spelling duration due to number of letters.

P300 speller spent time could have a relationship with the presented possible selections. Finally, the available BCI software do not support all EEG devices as well as using them may be difficult for non-technical users.

CONCLUSION

In this review, a detailed description of P300 speller paradigms was illustrated and some of its open issues were discussed. Researchers made huge improvement in engaging patients with the community by developing different P300 speller versions. However, according to the variety of P300 speller paradigms, many areas can be developed to enhance the spelling performance and improve patients' life quality.

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The Effectiveness of Thoracolumbar Fascia Kinesiotaping on Non Specific Chronic Low Back Pain in Selected Patients

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ABSTRACT

Non specific chronic low back pain(NSCLBP) is described as a tenderness or soreness, stretch or tension, and tightness or stiffness in the area of lower back, without any specific cause or pathology of that pain for 12 weeks. The part of fascial girdle is thoracolumbar fascia which covers lower portion of trunk and contain nerve endings and recurrent injury may contribute to low back pain.The objective of the present study was to observe the consequence of thoracolumbar kinesiotaping and trunk stabilization exercises on chronic lumbopelvic pain which was non - specific. About 100 male and female participants were included, in the strudy, having age group ranging from 18 to 60 years with nonspecific back pain for not more than 90 days. Selected participants were allocated to either of groups according to random sampling in two groups i.e. Control Group A (n=50), in which patients were given lumbar stabilization exercises and Experimental Group B (n=50) patients were given conventional physiotherapy with thoracolumbar fascia kinesio taping. Following this the assessment of patients was done by following methods: Oswestry Disability Index for functional disability, NPRS for pain, Tampa Scale for movement fear, Trunk ROM. The results demonstrated statistically significant improvement between both the groups in pain, kinesiophobia and trunk flexion, rotation to both side ROM (p=0.001) while all other variables was found to be non significant between both groups at the end of 4th week. This study concluded that there is efficacy of kinesiotaping for thoracolumbar fascia for patients having low back pain of chronic and non specific in nature.

KEY WORDS: KINESIOTAPE, KINESIOPHOBIA, NON-SPECIFIC LOW BACK PAIN, NPRS, ODI.

INTRODUCTION

One of the most commonest problem encountered in clinical physiotherapy and practice is back pain, (Caporaso et al.,2012; Dagenais et al.,2010). It is the main cause of restriction at workplace (Picavet et al.,2008). In back pain, usually the pain surrounds the lumbo-Sacro-coccygeal area around the pelvic muscles and may radiate to surrounding soft tissue structure up-to knee. Pattern of pain alters social life, functioning and activities of normal life (ADL). Low back pain which is non-specific can be seen with, before or after ossification of bones, (Kjaer et al., 2011 Aldera et al.,2020). Posture of human being is regulated by sensory tissue present in trunk and lower limbs, and any abnormalities in these tissues causes instability. Another possible cause of postural sway is acute pain inhibition. Due to pain proprioception is also affected and increase in pre-synaptic inhibition

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(Alexander et al., 2011; Mosley and Hodges, 2005; Brumagne et al., 2000, Hlaing et al., 2020).

Structures of spine like vertebral end plates are responsible for pain due to signal changes that disrupt and fissure the end plates as well as fatty degeneration of adjacent bone marrow occurs. One such structure that is also responsible is thoracolumbar fascia which has nerve supply and irritates back pain, (Secher et al., 2008; Schilder et al., 2014). The quality of patient's lives is affected due to this restriction affecting pain free movement and is ranked 6th globally causing great loss not only financially but also productively, (Chiarotto et al.2014, WHO Bulletin 2019). When mechanical traction is produced by muscular activity around the spine, TLF particularly respond and come into action for easy and effective load transfer. Tightness that occurs in fascia causes unnecessary stretch of intra-fusal muscle fibres, due to which alteration occurs in stretch-sensitivity in afferents fibres. It has ben reported that recurrent signs of trauma and inflammation in a piece of TLF of the patients with lower back pain, (Willard and Vleeming, 2012). For the advance treatment of low back pain which is chronic and non-specific in nature, a new approach was introduced in 1973 known as kinesio taping which covers the area which is injured and leads to decrease in pain, (Campolo et al., 2013 Lemos et al., 2015).

Dr. Kenzo Kase has developed these tapes which have similar properties like skin and has more elasticity than conventional bandage. The kinesio tape could be elongated till 40-60 % of original length. The tape elevates the epidermis due to which there is decrease in nociceptive stimulus. Accordingly, the Kenzo kase kinesio tape has multiple functions: it inhibits or facilitates the muscle function, decrease pain, support the surrounding structures, realign joints, improve blood circulation and lymphatic flow, improves proprioception and fascial tissue alignment, (Added et al., 2013 Kachanathu et al 2014). According to researcher's knowledge till date there is no such study conducted which has analysed the effect of thoracolumbar taping on pain, functional disability, kinesiophobia and lumbar range of motion in low back pain.

MATERIAL AND METHODS

In the present study, total 110 participants were volunteered, out of which 6 were not able to complete the protocol and 4 dropped out due to tape allergic reaction. Out of which 100 patients participated in which male (n= 52) and female(n=48) participants were included, having age group ranging from 18 to 60 years with nonspecific chronic back pain for 12 weeks or more and had an Oswestry Disability Index (ODI) score of more than 15%. Those subjects who suffered with spondylolisthesis, osteoporosis, history of spinal surgeries, pregnancy, psychiatric disorders, serious cardio-respiratory disease, spinal tumors or fractures, active or recent malignancy, spinal canal stenosis, large herniated disc, scoliosis were excluded, thus those who fulfilled eligibility criteria for the study, were only considered for the investigation, following the criteria of Magalhaes et al., (2013).

After thorough assessment and routine tests done by physician, patients were referred to the physiotherapy OPD for their treatment of nonspecific low back pain. The patients were examined and assessed by experienced researcher. Participants were informed and explained about the objective, treatment protocol, duration etc. of study. After written consent, participants were enrolled in the study. Selected participants were allocated to either of groups according to simple random sampling technique in two groups i.e. Control Group A (n=50), in which patients were given conventional physiotherapy which includes lumbar stabilization exercises and Experimental Group B (n=50) patients were given conventional physiotherapy with thoracolumbar fascia kinesio taping (Fig 1.1). Conventional physiotherapy treatment included group of exercise (lumbar spine stabilization exercise) and Hydro collateral pack (moist heat pack).

All patients received 8 treatment sessions group A (conventional) & group B (conventional physiotherapy and thoracolumbar fascia taping). After that baseline data (age, weight) were recorded by researcher before giving intervention. Following this the assessment of patients was done by following methods: 1) Oswestry Disability Index for functional disability. 2) NPRS for pain. 3) Tampa Scale for movement fear i.e. kinesiophobia. 4) Trunk ROM.

Fig 1.1 Thoracolumbar fascia Kinesio-tape

RESULTS AND DISCUSSION

110 patients participated in the study and 100 out of these underwent the treatment and completed the study. All the patients underwent a pre-assessment analysis which included age and weight measurement. 50 patients were given lumbopelvic stabilization exercises with hot pack in group A. 50 patients were given kinesio-taping along

with conventional treatment in group B on lower back for 4 weeks. The demographic data of the subject was as: Age:18-60 years, Mean for Group A is 37.54 ± 10.51and for Group B is 39.4 ± 10.45. Weight is Mean for group A = 55.32 ± 7.94 and for group B =56.66 ± 7.72.

Age, weight was assessed and analysis was done which revealed homogenecity in key demographic variables between the groups. Hence matched group were taken. The TAMPA score for movement fear, ROM of lumbar spine, numeric pain rating score for analysing pain and the Oswestry Disability Index score for analysing quality of life for all the patients belonging to the experimental group (KINESIOTAPING group) and the lumbo-pelvic stabilization group were taken prior applying the intervention. The mean scores of all the variables of both the groups were calculated before the intervention and after 2nd week and 4th week of intervention.

Table 1.1. Changes in NPRS between Group A and Group B

WEEK	(GROUP A)	(GROUP B)		
	Mean ± SD	Mean ± SD	t-value	p-value
WK 0	7.28 ± 1.78	7.78 ± 1.21	1.63	0.10^{NS}
WK 2nd	4.58 ± 1.21	4.88 ± 1.54	1.0	0.28^{NS}
WK 4th	2.34 ± 1.28	1.84 ± 1.49	1.79	0.01**

SD: standard deviation NS: not significant WK: week of intervention ** Significant at p≤0.05

The results did not show any significant difference in TAMPA between mean of group A (lumbar stabilization exercises) and group B (kinesiotaping) at 0 week,(t=-0.45, p= 0.65). However, at week 4, this difference was found to be significant (t= 4.45, p=0.00). (TABLE 1.2).

Table 1.2. Changes in TAMPA between Group A and Group B

WEEK	(GROUP A)	(GROUP B)		
	Mean ± SD	Mean ± SD	t-value	p-value
WK 0	48.56 ± 3.28	48.20 ± 4.59	0.45	0.65^{NS}
WK 2nd	43.92 ± 3.79	41.74 ± 5.07	2.43	0.01**
WK 4th	39.22 ± 4.85	34.88 ± 4.89	4.45	0.00**

SD: standard deviation. NS: not significant WK: week of intervention ** Significant at p≤0.05

The result showed non significant difference in flexion ROM between group A and group B (kinesiotaping), at 0 week (t=-1.72, p= 0.08).However, at week 4 flexion ROM for group A while that of group B (kinesiotaping),this difference was found to be significant (t= 3.45, p=0.01). (TABLE 1.3) Table 1.3 Changes in Flexion ROM between Group A and Group B.

Table 1.3. Changes in Flexion ROM between Group A and Group B

WEEK	(GROUP A)	(GROUP B)		
	Mean ± SD	Mean ± SD	t-value	p-value
WK 0	4.70 ± 1.14	4.33 ± 0.98	1.72	0.08^{NS}
WK 2nd	5.54 ± 1.13	5.82 ± 0.95	1.31	0.19^{NS}
WK 4th	6.52 ± 1.15	7.28 ± 1.02	3.45	0.01**

SD: standard deviation.WK: week of intervention.NS: not significant ** Significant at p≤0.05

The result showed no significant difference in right rotation ROM between mean of group A (lumbar stabilization exercises) and mean of group B (kinesiotaping) which was at 0 week (t=0.798, p= 0.42). However, at week 4, this difference was found to be significant (t= 3.50, p=0.01) (TABLE 1.4)

Table 1.4. Changes in Right rotation ROM between Group A and Group B

WEEK	(GROUP A)	(GROUP B)		
	Mean ± SD	Mean ± SD	t-value	p-value
WK 0	4.06 ± 0.83	3.93 ± 0.70	0.798	0.42^{NS}
WK 2nd	5.14 ± 0.77	5.02 ± 0.81	0.717	0.47^{NS}
WK 4th	6.17 ± 0.76	6.39 ± 0.74	3.50	0.01**

SD: standard deviation WK: week of intervention NS: not significant ** Significant at p≤0.05

The result showed no significant difference in left rotation ROM between mean of group A (lumbar stabilization exercises) and mean of group B (kinesiotaping) which was at 0 week (t=2.35, p= 0.21). However, at week 4,this difference was found to be significant (t= 2.58, p=0.01). (TABLE 1.5)

Table 1.5. Changes in Left rotation ROM between Group A and Group B

WEEK	(GROUP A)	(GROUP B)		
	Mean ± SD	Mean ± SD	t-value	p-value
WK 0	4.49 ± 0.84	4.12 ± 0.70	2.35	0.21
WK 2nd	5.51 ± 0.73	5.26 ± 0.72	1.66	0.09
WK 4th	6.65 ± 0.65	6.32 ± 0.60	2.58	0.01**

SD: standard deviation WK: week of intervention ** Significant at p≤0.05

The objective of the research was to check the clinical effectiveness of kinesiotaping for thoracolumbar fascia as a treatment intervention for patients having chronic non specific LBA in clinical outcome i.e. (pain & functional

disability) and physical function (range of motion &tkinesiophobia). NSLBP is the pain of musculoskeletal in origin and mechanical in nature of which clinical sign varies with the type of activities one is performing. NSLBP represents in form of pain, muscle tension, occasionally stiffness that is situated below the costal surface and on and over the gluteal folds inferiorly. It is commonest and mainly one of the self-limiting disorder.Many studies in literature having different research designs, researched to assess the relevance of manual therapy on LBP, which consisted of manual treatment alone or with electrotherapy modalities compared to exercises alone or with other interventions like consultation from medical professional with patient education; motor control exercises along with behavioural therapy; group exercises with CBT. Therefore, the researcher stated the design of the present research, with the application of fascial kinesio taping with exercise and hot pack in one group as compared to only conventional exercise with hot pack in other group.

The results demonstrated statistically significant improvement between both the groups in pain, kinesiophobia and trunk flexion, rotation to both side ROM. The result of the research concluded that thoracolumbar fascial kinesio-taping with or without conventional physiotherapy was effective in decreasing pain and increasing functional abilities. Patients who had received thoracolumbar fascial kinesio-taping in combination with hot pack and lumbopelvic strengthening exercises shows decreased pain and improved functional performance in comparison to one who received general physical therapy treatment only.

The findings of the present study revealed statistically significant differences between both the groups i.e. Group A (lumbopelvic strengthening exercises and hot pack) and Group B (Kinesiotaping and strengthening exercises with hot pack) and p = 0.01. Also pain was found to statistically improve within groups. It has been seen that KT puts pressure onto the skin or sometimes stretches the skin and it's that external load which may stimulate skin receptors which are sensitive to mechanical pressure which are large myelinated fibres specially and thus stops the transfer of pain in accordance to gate control theory. Melzack and Wall postulated that spinal cord consisted of a neurological "gate" that sometimes blocks pain signals or sometimes allows signals to transduce to the brain (Al Shareef et al.,2016). In contrast to the study by Paoloni and co workers, we observed decrease in disability which was statistically non-significant but kinesiophobia was highly statistically significant (p=0.00) which was measured using the TAMPA in both the groups of the present research.

In the present research the investigators had given core stabilization exercise for the participants with NSCLBP to stabilize and strengthen the deep spinal muscles which induces and improves trunk stabilization reducing pressure on the torso which leads to back pain reduction. Improvement in pain perception could be seen as a result of re-arrangement of the general control of the muscles of the spine which is placed deeply thus reducing the activity of many more superficial muscles (Bharti, Arora and Arora, 2015).

In present study researcher found statistically significant improvement (p=0.000) in forward flexion and rotation to both sides of lumbar spine between both the groups. There was no statistical improvement in extension and side-flexion both left and right. There was improvement in all the range of motions within groups. The improvement can be adjunct to the previous researches conducted which stated that KT influences sensorimotor function. Also, KT-induced change in muscle tone could result from stimulation of mechanoreceptors, which results in reflexive activation of motor units in the same muscle that was the source of the neural stimulus, (Lemos, Crolina and Gonsalves, 2014).

The thoracolumbar fascia is a stable structure because of the large flow of piezoelectric charges, which promote the deposition of collagen fibres and confers resistance to this tissues, and thus requires high tensions to be mobilized and stretched. Therefore, the present study found that a fascial correction technique and application without tension promoted changes in fascia mobility, allowed for discrete gains in lumbar flexibility, and had no significantly improved in extension and lateral flexion range of motion, (Lemos et al., 2009). Results showed statistically insignificant difference in extension and lateral flexion to both sides. This can be due to the short term results of kinesio tape application used in present study as inferences relative to long term effect can be seen in other studies, (Sarkar et al., 2018).

The therapists also used multimodal approach to the management of patients with NSLBP and had not used kinesio taping as an isolated intervention. This could be the reason that there is no statistical difference found in between the groups. This theory is supported by different authors in the literature. The limitation of the study is that the follow up was not done in study to assess the sustained effect of kinesiotape. Also, different age group analysis was not done. The future scope of this research could be following-duration of the study can be increased with regular follow ups. Results of the isolated kinesiotape can also be considered. Other variables like proprioception can also be taken to have a better perception of efficient pain management.

CONCLUSION

The present study concluded that there is efficacy of kinesiotaping for thoracolumbar fascia as an intervention for patients having low back pain of chronic and non specific in nature. The study states that experimental hypothesis is accepted.

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The Effect of Glucocorticoid Hormones on the Morphofunctional State of Red Blood Cells in Early Postnatal Ontogenesis

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ABSTRACT

The course of early ontogenesis is associated with changes in the morphology and physiology of red blood cells. Largely thanks to these changes, the body adapts to any changes in the environment that occur during the transition from the antenatal period to the postnatal period. Red blood cells at the beginning of ontogenesis always demonstrate greater functional stability, which gradually changes in the future. The red bone marrow and endocrine glands are actively involved in the regulation of erythropoiesis. A special place in this process is occupied by the adrenal cortex. The study confirmed that glucocorticoids in the early postnatal period of rat life are an important factor in the regulation of erythropoiesis involving T-lymphocytes. Erythropoiesis, previously considered at this age to be reactive to various stress factors, should be considered sensitive to their effects. This was demonstrated by increasing the concentration of red blood cells in the blood, increasing the number of red blood cells, discocytes and their average diameter after the administration of hydrocortisone to rats at the age of six days.

KEY WORDS: ERYTHROCYTES, RATS, ERYTHROPOIESIS, EARLY ONTOGENESIS, GLUCOCORTICOIDS, REGULATION.

INTRODUCTION

Red blood cells are the largest group of blood cells that realize the process of gas exchange in the body and the rheological properties of blood in vessels of any caliber (Zavalishina, 2018a; Glagoleva and Medvedev, 2018). Postnatal ontogenesis is marked by changes in red blood cells in their morphology and their characteristics (Vorobyeva et al., 2018). Largely thanks to these changes, the body adapts to any changes in the environment, primarily during the transition from the antenatal period to the postnatal period (Medvedev and Kumova, 2007a; Tkacheva and Medvedev, 2020). It is very important that red blood cells, especially at the beginning of ontogenesis, demonstrate greater functional stability, which gradually changes during development (Bikbulatova, 2018a; Vorobyeva and Medvedev, 2020).

It is recognized that red bone marrow, nervous system, endocrine glands, liver, spleen, kidney and organs of the gastrointestinal tract are primarily involved in the regulation of erythropoiesis (Medvedev and Kumova, 2007b).Most mammals are born with a fully formed neuroendocrine system. Further improvement of the function of individual links of this system or their consolidation occurs in the process of individual development of the organism. In particular, it is known that in rats whose intrauterine development proceeded during physiological pregnancy, by the time of transition to definitive nutrition, the hypothalamic-pituitary-corticoid system acquires high functional activity

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(Henning, 1978). In rats, this occurs at the end of the second week of life (Poland et al,, 1979). Nevertheless, it was found that physical activity throughout the pregnancy of rats leads to a delay in the natural dynamics of the morphofunctional features of red blood cells in postnatal ontogenesis in prenatally stressed offspring (Kartashev et al., 2017), which undoubtedly affects the respiratory function of the body.

As before, many issues of postnatal changes in the functional parameters of red blood cells remain insufficiently studied. As a result of this, to date, the available information on the mechanisms of restructuring the functional state of erythrocytes in postnatal ontogenesis is fragmentary (Karpov et al., 2020). The work on this problem was carried out by non-identical methods on different biological objects, in groups of different ages and incomparable. In this regard, the aim of the work was: to find out the role of corticoids in the formation of morphofunctional features of red blood cells in a growing organism.

MATERIALS AND METHODS

The study was conducted in strict accordance with the ethical principles of the European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes (adopted in Strasbourg on March 18, 1986 and confirmed in Strasbourg on June 15, 2006). In the experiment, white Wistar rats were used with a total of 50 animals. To obtain rat pups, females of approximately the same body weight of 180-200 g were selected; they were kept in cages of 8 individuals each. After preliminary getting used to the females, 3-4 males of about the same mass for mating were planted for one day. Fertilized females (this was determined by the presence of sperm in their vaginal smears) were placed in individual cells, kept under unlimited nutrition with sufficient drinking. From the first day after birth, the rat pups were distributed 8 for each lactating female.

On the sixth day of life, rat pups, when the natural level of corticosterone in their blood is still at a critically low level (Kozinets et al., 1977), they received a single injection of hydrocortisone (2.5 mg/100g body weight). This stimulated their body, outstripping the natural increase in blood levels of corticoid concentration, which is observed in rats in postnatal ontogenesis only at the end of the second week of life, reaching the level of adult animals (Henning, 1978). The control group received an injection of an equivalent amount of saline. Then, for 8 days every 24 hours, the rat pups of the control (n = 25) and experimental group (n = 25) evaluated the morphofunctional features of red blood cells.

To study the tested parameters in all cases, blood sampling in rat pups was carried out from the tail vein. The number of red blood cells in their blood was counted in the Goryaev's cell. The erythrocyte morphology was studied by scanning electron microscopy using a S-405 A HITACHI scanning electron microscope (Japan). To determine the percentage of different forms of red blood cells from each animal, 1-2 blood preparations were prepared and the number of scans was taken that provided at least 200 red blood cells.

On the scans, at least 1000 different forms of red blood cells were counted, followed by a percentage transfer. In accordance with the classification (Kozinets et al., 1977) discocytes were counted, discoid type erythrocytes were determined, erythrocytes with an outgrowth, with a crest, with multiple outgrowths were counted; assessed levels of a non-discoid type-red blood cells in the form of a mulberry, domed, spherical, in the form of a deflated ball, degeneratively altered. The average diameter of red blood cells was determined from the calculation of 100 cells.Quantitative data obtained during the study were processed using G.G. Avtondilova morphometric techniques (Avtondilov, 1990). The reliability of the results was determined by student's criterion.

Table 1. Dynamics of the number of red blood cells (in 1 mm³) in the blood of rats after a single injection of hydrocortisone at six days of age

The age of the animals (in days)	Duration of observation (day)	Control, n=25, M±m	An experience, n=25, M±m
6	Drug administration 0(initially)	2.78±0.08	
7	1	2.70±0.08	2.84±0.08
8	2	2.82±0.16	2.81±0.10
9	3	2.92±0.21	3.00±0.10
10	4	**3.54±0.20	**3.47±0.21
11	5	*3.21±0.16	*3.29±0.05
12	6	**3.81±0.05	**4.24±0.26
13	7	**3.54±0.14	**4.39±0.26
14	8	**3.84±0.20	**4.57±0.16
			p<0.05

Legend: significance of differences relative to the initial value in the group: * - p <0.05, ** - p <0.01; significance of differences between groups - p.

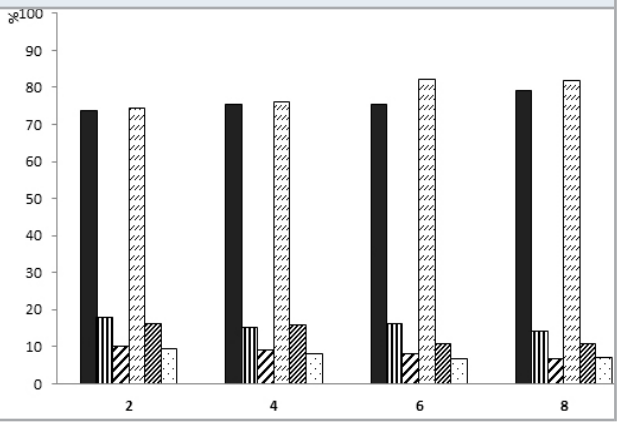
RESULTS AND DISCUSSION

The concentration of red blood cells in the blood of rat pups (table 1) practically did not change during the first three days of observation in both groups. On the fourth and fifth days after the exposure, both in the control and in the experiment, the level of erythrocytes increases relative to the initial one, no difference was found between the control and experimental groups. On the sixth day after exposure, the concentration of erythrocytes significantly increases compared with the previous days in both control and hydrocortisone-stimulated animals. However, in the latter, the increase

in the number of cells is more pronounced (by 28.9%). In the next day, the increase in the number of red blood cells in the blood of experimental animals continues. On the eighth day of the study, the concentration of red blood cells increases even more in the experimental group and becomes significantly higher than in the control.

Using scanning electron microscopy of red blood cells (Figure 1), it was found that the level of discocytes in the control group of animals increased on the eighth day of the experiment, while under the influence of exogenous hydrocortisone, quantitative growth of discocytes took place already on the sixth day. An increase in the level of erythrocyte-discocytes after the administration of hydrocortisone in the experimental group was accompanied by a decrease in the discoid and non-discoid types of red blood cells. Against the background of a general decrease in erythrocyte discoid forms on the sixth day, there is a significant decrease in erythrocyte disocytes with a crest and multiple outgrowths (Figure 1). In the group of non-diskoid cell types, by the fourth day of the study, there was a decrease in predominantly dome-shaped red blood cells in the control and in the experiment. On the sixth day in the blood of animals stimulated by hydrocortisone, they became significantly more than in control rat pups.

Figure 1: Changes in the morphology of red blood cells in rat pups after he was injected with hydrocortisone at six days of age. On the abscissa axis, days after exposure; on the ordinate axis, the number of cells in% of the total number taken as 100%.



Legend: I - discocytes, II - discoid group of cells, III - non-discoid group of cells in control animals; IY - discocytes, Y - discoid group of cells, YI - non-discoid group of cells in the experiment.

The average erythrocyte diameter (Table 2) did not significantly differ between the observed groups on the second, fourth, and eighth days of the study; on the sixth day only, in experimental animals it was significantly larger compared to the control ($p < 0.001$).

The results obtained suggest that the introduction of hydrocortisone to six-day-old rats by the end of the experiment increases the concentration of red blood cells, increases the number of red blood cells and their size and reduces the level of discoid and non-discoid red blood cells in animals.The results obtained in the study complement the available data on the structure and functions of rat erythrocytes in postnatal ontogenesis (Zavalishina, 2018b). The data confirm the information that at the beginning of ontogenesis, the number of red blood cells in the blood increases due to the high activity of erythropoiesis, which is clearly regulated by the endocrine system (Medvedev and Gamolina, 2008).

Table 2. Dynamics of the average diameter of red blood cells (in arbitrary units) in rats after the injection of hydrocortisone produced at six days of age

Observation groups	Duration of observation			
	2 day	4 day	6 day	8 day
Control, n=25, M±m	5.83±0.06	5.73±0.06	5.82±0.06	5.86±0.05
Experienced, n=25, M±m	5.74±0.06	5.62±0.06	*6.40±0.10 P<0.001	*5.82±0.05

Moreover, an increase in the concentration of red blood cells and the number of discocytes with a large average diameter (macrocytes) in rat pups with age is primarily associated with stimulation of glucocorticoid hormones. In this regard, it can be said that exogenous hydrocortisone at an early age in rats stimulates erythropoiesis, contrary to the opinion that the mechanisms responsible for it in the neonatal period are reactive with respect to specific stimulants erythropoietin (Filimonov and Tabarchuk, 1978) and corticosteroids (Balika and Kartasheva, 1982).

The obtained results to some extent contradict the prevailing opinion that in early postnatal ontogenesis, in particular in the first three weeks of rats (Filimonov and Tabarchuk,1978) and the first two months in dogs (Gorozhanin,1978), erythropoiesis is reactive. The authors who adhere to these points of view explain this by the fact that at this age the concentration of erythropoietin is at its maximum and, as a result, erythropoiesis is at its peak. Their experiments with the introduction of plasma animals with a high content of erythropoietin did not change the rate of incorporation of radioactive Fe into red blood cells (Filimonov and Tabarchuk, 1978). In this work, the action of endogenous erythropoietin after administration of hydrocortisone is excluded, which is observed in adult animals (Malgor et al., 1974; Morschakova et al., 1979; Romashko et al., 1985). Considering that under the conditions of the experiment, an increase in the concentration of erythrocytes after the administration of hydrocortisone occurs only on the sixth to eighth days, it can be assumed that the hormone exerts its effect on the hematopoietic cells not directly, but indirectly with the participation of some additional link (Zavalishina, 2018c).

Disclosure of this missing link in the course of corticosteroid-induced erythropoiesis has been helped by experiments in adult animals. In particular, an increase in the concentration of red blood cells in the experiment described in this article coincides with the timeframes identified earlier (Romashko et al., 1985). It was also previously shown that the effect of corticoids on erythropoiesis is carried out with the participation of the thymus (Moroz et al.,1984; Bespalov et al., 2018a). Graceful experiments (Dygay and Shakhov, 1989; Dygay et al., 1990) demonstrated that, in the chronic immobilization of adult mice, along with an increase in the concentration of glucocorticoids in the blood of animals, there is an increase in T-lymphocyte regulators (expressing lyt-1 + and lyt-2 + antigens on their surface) and their homing in bone marrow tissue with subsequent activation of erythropoiesis, which occurred on the sixth or eighth day, that is, at the same time as in our work with the introduction of hydrocortisone. Prior immobilization, administration of antithymocytic serum, thimectomy, or adrenalectomy inhibited this process. Substitution therapy of adrenalectomized animals restored this process.

It was also noted that an increase in the rate of erythropoiesis in response to the administration of exogenous glucocorticoids would not be observed if anterythropoietin serum was previously introduced (Malgor et al.,1974). This becomes clear when one considers the report of Lipton and Nathan, (1983) that, T-lymphocytes are regulators in bone marrow tissue due to their interaction with monocytes / macrophages with the release of lymphokine or monokine during this process (Bespalov et al, 2018b). Both substances, increasing the sensitivity of early erythroid committed precursors to erythropoietin, stimulate the proliferation and differentiation of committed precursors. It becomes clear that endogenous erythropoietin at the time of action of hydrocortisone in the experiment is the resultant factor in the activation of erythropoiesis.

Given the results, there is reason to adhere to the opinion of O.O. Romashko et al. (1985) on the presence in the organism of mammals of two corticoid-dependent erythropoiesis pathways. One of them is associated with the stimulation of erythropoietin synthesis (Malgor et al, 1974; Morschakova and Pashukov, 1982) and its subsequent action on cells sensitive to it (Medvedev and Kumova, 2007c). Another pathway of erythropoiesis is associated with the activation of its mechanisms sensitive to the action of T-lymphocytes (Bikbulatova, 2018b). This is convincingly demonstrated by Kalinina and Pashukov (1985) in the absence of adrenal glands in animals with high erythropoietic blood activity, where they observed a slight increase in reticulocytes and a lower level of their synthesis in the bone marrow.

It is noteworthy that on the eighth and ninth days of postnatal life, the concentration of red blood cells in humans (Leonova and Rapoport, 1989), productive animals (Vorobyeva and Medvedev, 2018; Tkacheva, 2020), reticulocytes in dogs (Balika and Kartasheva, 1982) and the rate of incorporation of radioactive Fe in rats increase (Filimonov and Tabarchuk, 1978). However, this phenomenon has remained unexplained. Apparently, the activation of erythropoiesis in the second week of fetal life is caused by a high level of hormones of the adrenal cortex in the mother's blood during birth stress. In this case, corticoids, as is known, freely pass from the bloodstream of the mother to the fetus (Zarrow et al., 1970; Mitskevich, 1978; McEven et al., 1986).

At this point, they include a mechanism for stimulating erythropoiesis with the participation of T-lymphocytes, and the effect of glucocorticoids is detected only after a week, when their basal level is significantly reduced (Henning, 1978; Poland et al., 1979; D'Agostino and Henning, 1982). The physiological significance of the corticoid-dependent activation of erythropoiesis during this period becomes clear, given that the newborn body has to adapt to completely new conditions and type of respiration.

CONCLUSION

Assessing the results obtained during the study taking into account the literature data, it can be concluded that glucocorticoids in the early postnatal period of rat life are an important factor in the regulation of erythropoiesis involving T-lymphocytes. Erythropoiesis, previously considered at this age to be reactive to various stress factors, was found to be sensitive to stimulation. This was demonstrated by an increase in the concentration of red blood cells in the blood, an increase in the number of red blood cells, discocytes and their average diameter after the administration of hydrocortisone to rats at the age of six days.

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Accuracy of Implant Placement Utilizing 3D Printed and Thermoplastic Surgical Guides: A CBCT-Analysis

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ABSTRACT

To achieve desirable functional and esthetic objectives in implant treatment, detailed case study, and planning is required. Since the introduction of 3D-printed technology into multiple fields, such as medicine and engineering, the use of this technology in dentistry is still to be explored. This study aimed to assess implant placement accuracy using two different implant surgical guides: thermoplastic (TP) and 3D-printed. Thirty acrylic resin mandibles missing the second premolar were fabricated with stereolithography (SLA) based on data from the CBCT scan. 15 TP and 15 3D-printed guides were constructed for the placement of the implants in relation to the mental foramen, and virtual implant apex distance from the mental foramen was set as 3.18 mm. 30 dummy 3.5 x 8mm implants were installed into the replica jaw models. Post-placement CBCT scans were compared to the virtual implant placement in relation to the mental foramen with the actual implant placement. The mean±SD of the implant distance to mental foramen for the 3D and TP guides was 3.12±0.36 mm and 2.52± 0.83 mm (P<0.05); respectively. The deviation apex of the implant for the 3D-printed and TP guides was 0.92±0.14 mm and 1.57±0.45 mm (P<0.001); respectively. The angular deviation of the 3D-printed and TP guides was 3.33 ±0.86o and 4.03±1.13o, respectively. Based on this study, the 3D printed guides were more accurate than the TP guides in terms of implant placement accuracy in relation to an important landmark and 3D implant placement.

KEY WORDS: CBCT; DENTAL IMPLANT; 3D-PRINTED GUIDE.

INTRODUCTION

Since the beginning of the implantology era, clinicians paid a great effort to improve implant performance and to minimize adverse effects and procedural errors. The accuracy of surgical implant placement and its relation and proximity to vital structures has always been a concern of the practitioners.Methods such as the use of a clipper measurement system and cone-beam computed tomography (CBCT) in the treatment planning of implant treatment have been used, and both showed some degree of inconsistency (Chen et al., 2008). The traditional method of placing an implant with the help of a surgical guide is to construct a radiographic stent, then transforming it to a surgical guiding device after taking CBCT (Misch 2004). However, the use of the traditional method has complicated lap procedures, limited accuracy, and tricky implant fixture surgical placement (Nickenig and Eitner, 2007). To overcome the shortcoming of the traditional method, computer-aided design/computer-assisted manufacturing (CAD/CAM) technology was introduced in 1971, and it has been used more often recently in dentistry (Kim et al., 2018 Abdou and Lau 2020).

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In CAD/CAM system, CAD undergoes a process of scanning and designing, and CAM systems are divided into subtractive manufacturing (SM) and additive manufacturing (AM) (Kim et al., 2018). Solidified blocks are milled in subtractive manufacturing, which provides high accuracy (Kim et al., 2018). However, the waste material cannot be reused; different burs are used for different blokes, and due to bur erosion, errors may occur, which makes the subtractive manufacturing costs high (Kim et al., 2016a, Ortorp et al., 2011 Abdou and Lau 2020).

A potential solution to the problems of subtractive manufacturing is the additive manufacturing, which involves high-intensity laser as an energy source to melt and fuse selective regions of powder, layer-by-layer free-form to build up a 3-dimensional component according to the computer-aided designed structure using different materials (Kim et al., 2017b). For the individual layers to be generated, the CAD data are uploaded to the selective laser melting (SLM) machine for the production of components, the micro-stereolithography (μ-SLA) files have to be processed by the software, such as Magic, to provide support to structures for any overhanging features (Joo et al., 2016). 3D-printed surgical templates are printed with the use of digital light processing (DLP) which uses a layered, ultraviolet (UV)-cured resin material, and only each UV resin layer is only a few microns thick, resulting in a highly precise surgical template (Kim et al., 2017a, Lee and Cho, 2003). Another great advantage is the commercially available software, which allows clinicians to interact with CT scan data. The combination of CT-based treatment planning and (CAD/CAM) of surgical templates allows clinicians to plan treatment in advance (Kim et al., 2016b Abdou and Lau 2020).

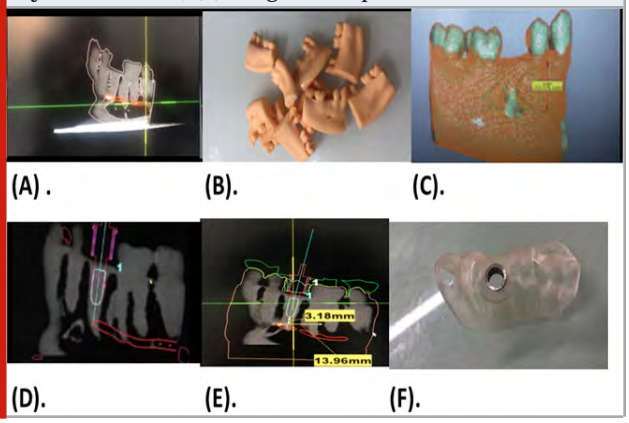
This technology is a revolutionary method that is expected to open new and better approaches of treatment (Schneider et al., 2009). 3D-printing was first used as rapid prototyping and rapid tooling technology. Dentistry use of single, personalized objects made a strong relationship between the two fields. Dental labs already started using 3D-printing in accurately manufacturing crowns, bridges, plaster/stone models, orthodontic, and surgical appliances (Kuhl et al., 2013). 3D-printing was also used in manufacturing single titanium dental implants with a promising success (Buser et al., 2012 Abdou Lau 2020). Therefore, this study conducted to assess the accuracy of implant placement using 3D-printed and thermoplastic surgical guides.

MATERIAL AND METHODS

Data acquisition: This study was conducted to evaluate the accuracy of implants placed using two different guided implant surgery materials: thermoplastic versus 3D-printed. Thirty acrylic resin mandibles were fabricated with stereolithography (SLA) based on data from the cone beam computerized tomography (CBCT) scan, which were converted into a Digital Imaging and Communications in Medicine (DICOM) file. The mandible was modified digitally by removing the lower right second premolar by using (zarokhan modifier) and converted to STL file before printing. The planed model of the mandible jaw was then exported for printing and was printed using (FORMALAB2) 3D-printer. Registration of the mental foramen was done clinically and was marked by a Standard composite cube to be accurately located, and a CBCT scan was taken for the resin mandibles.

Surgical plan and template fabrication: The modified CBCT scanned data was exported to an implant planning software (ProDigiDent, ImplaStation for Windows x6464 Bit Beta Version) for planning a specified implant position in relation to the mental foramen. A digital plaster model was then imported and superimposed with the CBCT data and exported to an STL file for the fabrication of the surgical template. All templates were printed using (FORMALAB2) 3D-printer using UV cured acrylic-based resin in 16 um layers. A total of 15 3D-printed surgical guides and 15 thermoplastic surgical guides had been made for the placement of the implants in relation to the mental foramen by the same lab technician and virtual implant apex distance from the mental foramen was set as 3.18 mm. One (ASTRA TECH) 3.5 x 8 mm implant was placed per guide and replica jaw model. Postsurgical CBCT scans were done to compare the virtual implant placement in relation to the mental foramen with the actual implant placement.

Figure 1: Data acquisition and surgical plan. (A) Mandibular digital model. (B). Resin model, (C). digitel resin model, (D) planned implant position, (E). surgical template created by the software, (F) Surgical template.



Statistical Analysis: Regarding the statistical analysis, Independent Samples Test and post hoc analysis were utilized with a p-value ≤ 0.05 that considered the cut-off point of statistical significance.

RESULTS AND DISCUSSION

All CBCT scans of the surgical template fitted on the plaster model were performed by the same operator. There were 15 implants planned in the software, and no templets were fractured in this study. There were significant differences in all outcome variables (i.e., implant apex to mental foramen, buccal axial section,

and lingual axial section) between the thermoplastic and the 3D-printed implant positions. The mean ± standard deviations (SD) of the implant relation to mental foramen in the 3-D Printed guides were 3.12±0.36 mm, respectively. On the other hand, the mean and SD of thermoplastic guides were 2.52±0.83 mm. The deviation at the apex of the implant of the 3D-printed guides was 0.92±0.14 mm and 1.57±0.45 mm for the thermoplastic guides. For the implant, angular deviation of the 3D-printed guides was 3.33±0.86o and 4.03±1.20o for the thermoplastic guides, respectively.

Table 1. Mental Foramen Distance, Apex Deviation Distance. Head Deviation Degree.

	T-Test Method		Group Statistics N	Mean	SD	p-value
Mental Foramen Distance	Thermoplastic Guide		15	2.52 mm	0.83	.0.019
		3D Guide	15	3.12 mm	0.36	0.000
Apex Deviation	Thermoplastic Guide		15	1.57 mm	0.45	
		3D Guide	15	0.92 mm	0.14	
Head Deviation	Thermoplastic Guide		15	4.03o	1.20	0.076
		3D Guide	15	3.33o	0.86	

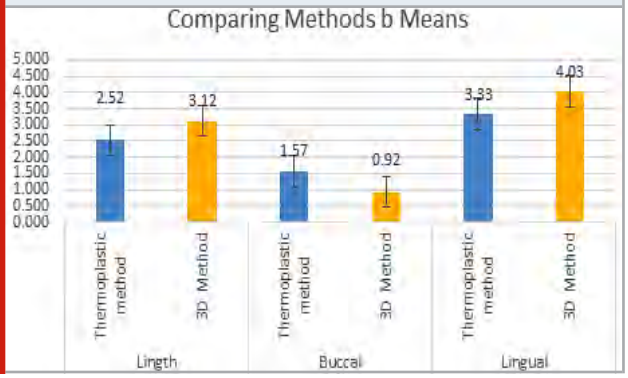


Figure 2: Surgical guide comparison. (Length). Implant apex to mental foramen (Buccal). Buccal axial section, (Lingual), Lingual axial section

The computer-assisted surgical guide combines the computer 3D-image, which helps identify the anatomical structures of the bone, together with the prosthetic information, in order to find the ideal region to place the implant and to minimize the damage to vital structures. The results of this study showed that; the mean distance for the implant placed using the thermoplastic surgical guide is 2.5±0.83 mm; in contrast, the 3D-printed guide showed a 3.12± 0.36 mm. This difference was highly significant and constant for the 3D-printed guides. This indicates that the 3D-printed guides are more accurate and safe to be used in areas with vital structures. This is in agreement with the findings of a group of researchers who suggested that laboratory-fabricated surgical guides using CBCT data may be reliable in implant placement under prosthodontic considerations in partial edentulism (Behneke et al., 2012)

The deviation at the apex of the 3D-printed guides was 0.92 mm and 1.57 mm for the thermoplastic guides. This in consistent with Bell et al. (2018), who found that thermoplastic showed a difference between 3D-printed guides (0.76 mm) and thermoplastic guides (1.60 mm). In addition, Abduo & Lau (2020) reported 0.71 mm apex deviation for 3D-printed guides, while pilot-guides had 1.14 mm.For the angular deviation of the 3D-printed guides were 3.33o and 4.03o for the thermoplastic guides, respectively. This in agreement with Bell et al. (2018), who reported a deviation of 2.36o for 3D-printed guides and 3.40o thermoplastic guides. Recently Abduo & Lau (2020) found that fully guided had 2.42o deviation while pilot-guided had 4.65o deviation.

CONCLUSION

Based on this study, the 3D printed guides were more accurate than the TP guides in terms of implant placement accuracy in relation to an important landmark and 3D implant placement.

Disclousre: This is study is registered at the Research Center of College of Dentistry King Saud University with registration number CDRC IR 0318. The authors claim to have no financial interests, either directly or indirectly, in the products or information listed in the article.

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Shelf Life Improvement of Lucuma (*Pouteria lucuma*) Fruit Under N-Succinyl Chitosan Incorporated with Turmeric as Edible Coating

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ABSTRACT

Turmeric is commonly known as a safe, nontoxic, bioactive ingredient. N-succinyl chitosan is a promising chitosan derivative developed particularly for biomedical, food and packaging applications. Edible coating not only creates good barrier to vapor and oxygen during fruit preservation but also increases its safety due to their natural bioactive component. Lucuma (*Pouteria lucuma*) is an excellent fruit due to its intense yellow colour and unique sense. It is a rich source of in carotenoids; minerals, vitamins, dietary fibres, triterpenes, phenolic subtances with numerous biomedical and pharmaceutical advantages. The main obstacle of lucuma fruit storage is its high perishibility leading to loss of firmness, soluble dry matter, carotenoid and total phenolic. We attempted to examine the effect of N-succinyl chitosan incorporated with turmeric (0.45%: 0.05%, 0.40%:0.10%, 0.35%: 0.15%, 0.30%: 0.20%, 0.25%: 0.25%) and storage temperature (8, 12, 16, 20, 24oC) to the weight loss (%), firmness (N), total soluble solid (oBrix), carotenoid (mg/100g) and total phenolic (mg GAE/100g), overall acceptance (sensory score) in lucuma (*Pouteria lucuma*) fruits during 15 days of storage. Results demonstrated that N-succinyl chitosan incorporated with turmeric (0.35%: 0.15%,) and storage temperature at 16oC could effectively maintain physicochemical, phytochemical and organoleptic attributes of lucuma (*Pouteria lucuma*) fruit for 15 days. Edible coating created semi-permeable film to successfully delay ripening and extend the storage stability of lucuma fruit.

KEY WORDS: CAROTENOID, FIRMNESS, LUCUMA, N-SUCCINYL CHITOSAN, TOTAL SOLUBLE SOLID, TOTAL PHENOLIC, TURMERIC, WEIGHT LOSS.

INTRODUCTION

N-succinyl chitosan is an acyl derivative of chitosan that is biocompatible, biodegradable, bioadhesive, water soluble in acidic as well as in alkaline media, long-term retention (Kato et al. 2000; Yan et al. 2006). It is potentially robust and is rich in reactive functional (-NH2, -OH, and -COOH) groups. It also has excellent moisture absorption and retention property, superior chelating ability, significant apoptosis inhibitory, enzyme immobilization, strong antimicrobial and antioxidant activity, and greater bioactivity than its parent molecule chitosan (Hasegawa et al. 2001; Luo et al. 2010; Zhang et al. 2014; Prashanth and Tharanathan 2007; Zhou and Wang 2009; Kong et al. 2010; Sun et al. 2006; Fan et al. 2010; Inta et al. 2014; Vinsova and Vavrikova 2011; Guo et al. 2008).

Turmeric has different biological properties, such as anti-inflammatory, antioxidant, and anti-carcinogenic attributes (Mahmoud et al., 2019). Turmeric exhibits safe,

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nontoxic, broad range of biological attributes (Boruah et al., 2012; Nazari et al., 2017; Shaikh et al., 2009). Turmeric oil consists of secondary metabolites that can act as antimicrobial agent. Nano-emulsion coated with chitosan is a promising delivery system to promote the applications of curcumin in functional food and beverage (Jinglei et al., 2016). Turmeric is includeded in the chitosan coating as innovation based on the possible synergy effect of these two components to improve the storability of strawberries after postharvest (Noorsuhana et al., 2018).

Lucuma (*Pouteria lucuma*) fruit belongs to Sapotaceae family (Marianela et al., 2019). Its pulp has an intense yellow pigment, sweet pleasant feeling and specific flavor. Its sweet taste is exploited to be used as natural food sweetener (Banasiak, 2003). Its pulp has a low moisture content but high protein and reducing sugar (Erazo et al., 1999; Brizzolari et al., 2019). It contains a great variety of carotenoids; minerals, vitamins, dietary fibres, triterpenes, phenolics beneficial for human health (Rojo et al., 2010; Fuentealba et al., 2016; Albena et al., 2019). Lucuma pulp has been widely supplemented to n various food applications (Dini, 2011). Lucuma fruit has been considered as one of super fruits (Mukta et al., 2017) due to its ability to cure antihyperglycemia and antihypertension (Marcia et al., 2009), wound healing properties (Leonel et al., 2010). N-succinyl chitosan is normally utilized in biomedical but rarely applied in fruit coating, especially luuma fruit. The objective of the present study was to examine the effect of N-succinyl chitosan incorporated with turmeric and storage temperature to the weight loss, firmness, total soluble solid, carotenoid, total phenolic, overall acceptance in lucuma (*Pouteria lucuma*) fruits during 15 days of storage.

MATERIAL AND METHODS

Material

We collected lucuma (*Pouteria lucuma*) fruits in Tien Giang province, Vietnam. They were cultivated following VietGAP to ensure food safety. After harvesting, they were quickly conveyed to laboratory for experiments. These fruits were washed under tap water to remove foreign matter. *Beside lucuma* we also used other materials during the research such as chitosan, turmeric, acetic acid, succinic anhydride, acetone, NaoH, ethanol, methanol, sodium carbonate, gallic acid. Lab utensils and equipments included biuret, refrigerator, digital weight balance, penetrometer, refractometer, spectrophotometer.

Methods: Chitosan incorporated with turmeric (0.45g: 0.05g, 0.40g:0.10g, 0.35g: 0.15g, 0.30g: 0.20g, 0.25g: 0.25g) was dissolved in 100 ml of 1 % acetic acid and stirred for 30 min at 50 °C. Then, 50 ml methanol was supplemented to dilute the solution followed by dropwise addition of already dissolved 2.0 g succinic anhydride in 25 ml acetone. The mixture was stirred at 1200 rpm at 50°C for 24 hours. After 24 hours, reaction mixture was diluted with excess 1 M NaOH solution until clear solution was obtained. The clear solution was kept under stirring for 24 hours at 50°C. Then, ethanol was added to precipitate the product followed by filtration to separate the precipitates. The precipitates were purified by redispersing in ethanol for 24 hours and washed with ethanol and acetone several times to remove the excess of reactants. Pure product was dried in vacuum oven for 8 hours at 50°C (Shahid et al. 2019). Lucuma (*Pouteria lucuma*) fruits were dipped in the film forming dispersions for 45 seconds and air-dried for 30 minutes at ambient temperature. All samples were kept in storage temperature (8, 12, 16, 20, 24oC) in 15 days. The weight loss (%), firmness (N), total soluble solid (oBrix), carotenoid (mg/100g) and total phenolic (mg GAE/100g), overall acceptance (sensory score) in lucuma (*Pouteria lucuma*) fruits were evaluated.

Physico-chemical and sensory evaluation: Weight loss (%) was evaluated by the following formula: Weight loss (%) = [(A–B)/A] x 100 where A indicates the fruit weight at the time of harvest and B indicates the fruit weight after storage intervals. Firmness (N) was measured by penetrometer. Total soluble solid (oBrix) was determined by handheld refractometer. Carotenoid (mg/100g) was evaluated by near infrared spectroscopy. Total phenolic content (mg GAE/100g) was estimated using Folin-Ciocalteu reagent procedure. Sensory score was evaluated by a group of 13 panelists using 9 point-Hedonic scale.

Statistical analysis: The experiments were run in triplicate with three different lots of samples. The data were presented as mean±standard deviation. Statistical analysis was performed by the Statgraphics Centurion version XVI.

RESULTS AND DISCUSSION

Physicochemical, phytochemical characteristics of raw Lucuma (*Pouteria lucuma*) fruit The physico-chemical, phytochemical properties of fresh Lucuma (*Pouteria lucuma*) fruit were evaluated. Results were mentioned in table 1. It's clearly noticed that Lucuma was a great source of carotenoid as well as total phenolic content.

Table 1. The chemical compositions in fresh *Lucuma* (*Pouteria lucuma*) fruit

Parameter	Firmness (N)	Total (oBrix) soluble	solid Carotenoid (mg/100g)	Total phenolic (mg/g)
Value	8.74±0.03	21.39±0.02	37.25±0.00	69.32±0.01

Note: the values were expressed as the mean of three repetitions;

Effect of different N-succinyl chitosan concentrations to weight loss, firmness, total soluble solid, carotenoid, total phenolic and overall acceptance of Lucuma *(Pouteria lucuma)* fruit: Effect of N-succinyl chitosan

incorporated with turmeric (0.45%: 0.05%, 0.40%:0.10%, 0.35%: 0.15%, 0.30%: 0.20%, 0.25%: 0.25%) to weight loss (%), firmness (N), total soluble solid (oBrix), carotenoid (mg/100g), total phenolic (mg GAE/100g) and overall acceptance (sensory score) was assessed. All samples were kept at 24oC for 15 days. Results were presented in figure 1. It's obviously noticed that edible coating by N-succinyl chitosan incorporated with turmeric (0.35%: 0.15%) significantly ($P< 0.05$) maintained weight loss (%), firmness (N), total soluble solid (oBrix), carotenoid (mg/100g), total phenolic (mg GAE/100g) and organoleptic score of treated fruits. The appropriate edible coating would minimize respiration rate, weight loss respiration, oxidative reaction, as well as physiological disorders. Therefore fruit shelf life would be increased respectively. In one report, chitosan-starch coatings enhanced with turmeric essential oil were effective on preserving strawberry (Yusof et al., 2020).

Figure 1: Effect of N-succinyl chitosan incorporated with turmeric (%:%) to weight loss (%), firmness (N), total soluble solid (oBrix), carotenoid (mg/100g), total phenolic (mg GAE/g), overall acceptance (sensory score) of lucuma (Pouteria lucuma) fruit during preservation (24oC in 15 days)

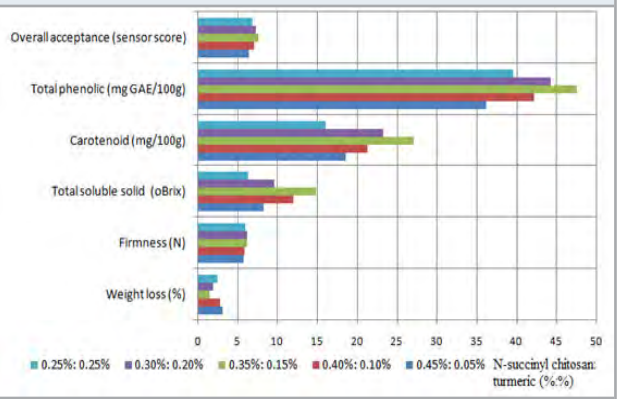
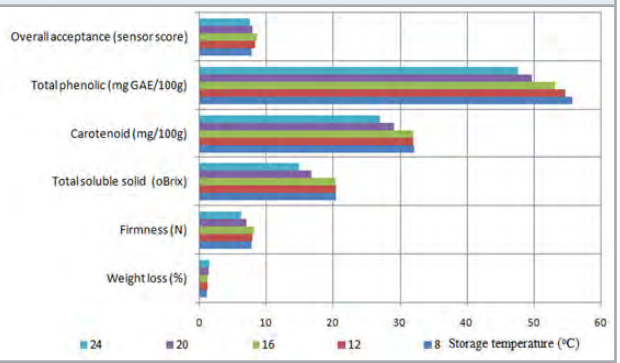


Figure 2: Effect of storage temperature to weight loss (%), firmness (N), total soluble solid (oBrix), carotenoid (mg/100g), total phenolic (mg GAE/g), overall acceptance (sensory score) of Lucuma (*Pouteria lucuma*) fruit



Effect of storage temperature to weight loss, firmness, total soluble solid, carotenoid, total phenolic, overall acceptance of Lucuma *(Pouteria lucuma)* fruit: After finding the appropriate ratio of N-succinyl chitosan: turmeric coating concentration (0.35%: 0.15%); the physicochemical, phytochemical and overall acceptance of Lucuma (*Pouteria lucuma*) fruit were also evaluated by the effect of different storage temperature (8, 12, 16, 20, 24oC) in 15 days of storage. Results were shown in figure 2. Optimal storage temperature for Lucuma (*Pouteria lucuma*) preservation was noticed at 16oC. The factors contribute to the physicochemical and phytochemical degradation in vegetable and fruit were mostly due to the moisture reduction caused by respiration and transpiration processes. By keeping a commodity at low temperature, respiration was reduced and senescence was also delayed, thus extending storage life (Halachmy and Mannheim, 1991).

CONCLUSION

N-succinyl chitosan is an amphiprotic derivative obtained from the N-acylation of chitosan. It has extraordinary biocompatibility, significantly increased aqueous solubility in acidic and basic media without altering the biological characteristics, appreciable transfection efficiency, and the capacity to stimulate osteogenesis. Lucuma is a good source of biologically active substances especially carotenoid, an excellent antioxidant activity with antihyperglycaemic characteristic. This research has successfully found out the appropriate conditions for maintaining Lucuma (*Pouteria lucuma*) fruit quality by N-succinyl chitosan incorporated with turmeric as edible coating, storage temperature. Turmeric incorporated with N-succinyl chitosan coating createed a synergistic effect to improve the stability of lucuma fruit.

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Conflict of Interest: The authors declared that present study was performed in absence of any conflict of interest.

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Applications of Microchip Based Technology in Modern Health Care: A Mini Review

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ABSTRACT

Human beings are mainly composed of different cells / tissues / organs which carry out various physiological roles representing a complex functional system. Now a days, it is difficult to predict the interactions between organs, including cells or tissues using in vitro cell culture approaches, as a result, animal tests should be conducted, pertaining to predict pharmacokinetics. So, major challenges as given through implantable technologies except the biomedical arena, showed some ignorance in mid-1990s. Few scientists have claimed some benefits about the usage of these medical-related technologies to patients, who have suffer from curable diseases or illnesses. Even today, scientists argue that this technology can be dangerous for the society at large, if applied incorrectly. Now, chip based technology has taken over the last few years. Various uses of microchips are applied especially in the field of medicine and human health care as well. The major benefits for this technology are reduced costs, low sample volumes, ease of use and precise results. This technology is to be used extensively in point-of-care diagnostics in less-developed countries. These chip based devices should be applied and are used to observe continuous or linked through various pharmacokinetics or immuno biological processes such as absorption, distribution, excretion, metabolism of various drug administration routes. Microchip technologies have all been expanded very rapidly and are coupled with various types of detection techniques which may be suitable especially for high-throughput screening including detection and mechanistic study of drugs. In this review article, we have discussed the importance and need of microchip technology for potential future development in the field of health care and diagnostics.

KEY WORDS: MICROCHIPS, TECHNOLOGY, MEDICAL SCIENCE HEALTH CARE.

INTRODUCTION

In spite of various new drug discoveries are reported but a major healthcare problem is still there because of non-existence of drugs for many infectious or non-infectious diseases. In addition, existing drugs are available somehow in the market but do not work in some patients and showed some side effects of drugs as well but considering as one of the leading causes of morbidity. Due to this reason, conventional studies were applied related to animal models for immunobiological research. In contrast, animal experimental based studies, raise some ethical issues and require new ways to improve drug development but their major drawback is the time-consuming process. Now a days, chip based technology is a recent contribution pertaining to solving various health care problems, especially in human systems, (mimicking

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human physiology with standard cell cultures on plastic substrates). Thus the existence of chips on organs which represent human tissues or cells of healthy individuals or patients under normal, or pathological conditions like those in the human body has been successfully developed (Kcomt 2019).

Healthcare is one of the most essential components related to human life. Now a days, number of chronic illness cases have increased enormously and have showed significant effects on modern healthcare (Kcomt, 2019). In spite of this, demand for researchers, doctors including paramedical staff members (nurses, microbiology and biotechnology) is extremely very high. In short, each country has its healthcare system, but there is some positivity and negativity as well (Borges et al., 2019). Those countries which are spending a lot of money on healthcare may have their other industrial sectors affected, while those countries which are spending less amount of money on the population's health, suffer more, (Lehnert et al, 2011; Gupta et al., 2018; Borges et al., 2019).As a result, this means healthcare can contribute more to the society which directly or indirectly is influencing human health. In short, healthcare arena is one of the most diversifying areas regarding preventive and personalized medicine (Guan, 2019). The measurement of health status is much more essential and advising patients including healthcare professionals on the most appropriate preventive, or curative measures (Hazarika, 2013; Borges et al., 2019; Guan, 2019).

In spite of this, our ability to evaluate our health status is totally hampered regarding complexity including cost, size and number of instrumentations that are required to acquire the data, and then analyze it. This type of data is much more required that transforms into more actionable information (Hazarika, 2013; Dikid et al., 2013). The major objective behind this technology is to improve its efficacy and morbidity using microchips where device manufacturers incorporate various technological advancements into medical implants. Recently, microchip based technology represents one of the new types of technological investmnents which is capable of drug release (on demand basis) over a long period, (Haitao et al, 2019; Kmiec et al., 2019).

The chip based technology has showed great potential and is more transformative related to modern healthcare systems. As per the literature, human studies involving microchips (e.g. dialysis) have been used and can be used in treating several other diseases such as diabetes and hypertension (Santini et al., 2000; Sharma et al., 2006; Rajgor et al., 2011; Haitao et al, 2019; Kmiec et al., 2019). In addition, microchips involvement is also seen to create artificial type of glands. The major role of hormonal regulation within the body which is directly associated with dysfunctional glands which helped in both controlling current disease state disorders and preventing them via other hormonal prompted disorders (Rajgor et al., 2011; Haitao et al, 2019; Kmiec et al., 2019).

The major challenge for biotechnological and pharmaceutical companies is regarding the testing of samples. In general, more than 92 percent of drugs undergo animal testing i.e. preclinical tests which is mandatory and required information but fail them to enter into the market (Akhtar, 2015). In an effort to reduce the budget cost, industries or institutes mostly relies on in vitro cell cultures and cell-based assays which is applied especially in biomedical research, pharmaceutical development and toxicity testing. In other words, in vitro cell culture is one of the most essential component of cell biology but its technology in advancement level may be declined as compared with the fields of genomics and proteomics and also through high-throughput testing of biochemical (Hartung, 2000; Coecke et al., 2005; Akhtar, 2015).

Scientists have developed some alternative methods regarding testing of animals pertaining to improve the assay validity and throughput capability is called organ-on-a-chip technology (3D human living cell cultures that are cultivated in a dynamic microchip environment under controlled condition that maintain human tissue functionality or mimic organ dysfunction). So, these disease-specific human cell types can be used to establish individual micro tissues with physiologic cellular behaviour, organ-on-a-chip technology can also be used for in vitro disease modelling (Hartung, 2000; Coecke et al., 2005; Akhtar, 2015). In this regard, device manufacturers incorporate some chemicals or drugs in microchips (solid silicon based) especially seen in medical implants (Haitao et al, 2019; Kmiec et al., 2019).

The designing of microchip is very simple and easy to manufacture but substrate contains multiple type of reservoirs which is capable of holding many chemicals in the form of solid, liquid, or gel form (Haitao et al, 2019; Kmiec et al., 2019). Overall, thus microchip is biocompatible and easy to implant in the human body. Each reservoir is mainly capped with a conductive membrane (i.e. gold) and wired with final circuit system which is controlled through microprocessor. In microchips, gold is used as standard membrane filter model because of its low reactivity with other substances, and also resists spontaneous corrosion in most of the solutions over the entire pH range. In addition, gold is considered as biocompatible material and presence of a small amount of chloride ion creates an electric potential region which favors the formation of soluble gold chloride complexes. In other words, this microchip may be considered as first device of its kind which enables the storage of compounds (one or more) inside the microchip in any form along with compound release on demanding basis, and with no moving parts (Haitao et al, 2019; Kmiec et al., 2019).

Background: History of microchips: is one of the most important parts of computer technology today and considered as a unit of packaged computer circuitry (also called as integrated circuit). This microchip is manufactured from one of the materials i.e. silicon at a very small scale.The process of creating a microchip

only begins with a type of sand called silica sand, which consists of silicon dioxide. In short, silicon may be the best candidate material for manufacturing process of semiconductor and always remain pure before used during manufacturing (Haitao et al, 2019; Kmiec et al., 2019).

- Introduction of first microchip in the year 1950s, but its size is much smaller as compared to fingernail and its cost so less than a dollar
- History of microchips started in the early 1950's when Geoffrey W. A. Dummer introduced type of software called them as microchips (immeasurable amounts of information and carry out various tasks, like today with I pods, computers, etc.).
- After 1958, researchers started making all sorts of sizes for microchip.
- In 1961, first commercially chips are available from Fairchild Semiconductor Incorporation. So, computers began to be made with microchips instead of individual transistors and their accompanying parts.
- In 1967, Jack Kilby invented the calculator (using microchips) and also won many awards including patents as well.
- Robert Noyce filed and published several patents and founded the company Intel, person is responsible for this invention i.e. microprocessor in 1968.
- In 1974, Roland Moreno from France who patented this technology called as smart cards or integrated circuit cards or embedded chip-on-a-card technology. This technology should easily identify the cardholder.
- In 1998, first demonstration of microchip implantation was achieved by Professor Kevin Warwick in case of human for identification and tracking purposes.

Chips are usually made in fabrication plants, multibillion-dollar investment and these are called as fabs. So, these fabs ultimately melt and refine sand to produce silicon ingots (99.9999% pure single-crystal). In addition, these saws slice the ingots and converted them into wafers (thick as a dime and several inches in diameter). Finally, wafers are cleaned and polished, and each one is used to build multiple chips (Chien et al., 2007; Morrison and Martin, 2007; Wu et al., 2015). Overall, all major steps are done especially in a clean rooms environment where precautions are taken pertaining to prevent contamination e.g. dust and other foreign substances. In addition, strategies are proposed with respect to human healthcare as shown in Table 1 (Chien et al., 2007; Morrison and Martin, 2007; Wu et al., 2015).

Table 1. Strategies for chip based technology and human health care

S.No.	Strategies
1	Design products, chip based which enable better clinical outcomes
2	This technology is applied in low-income based countries pertaining to reduce the program cost and implement its value of engineering to optimize product cost or development.
3	To build innovative ideas or solutions which may deliver high-quality, patient-centric care including improve access pertaining to advance diagnostics for millions of peoples.

Traditional methods for the fabrication of microchips:

- Photoresist- wafer, silicon dioxide, non-conducting layer is deposited on the surface and covered with photosensitive material called as photoresist. When photoresist is exposed with ultraviolet light which ultimately hardens that particular area exposed to the light (Faria-Briceno et al, 2019).
- Photolithography- Hardened process of photoresist called as photolithography, using different masks, followed by more etching and doping and repeated hundred times for the same chip and then finally converted into a more complex type of integrated circuit at each step (Basara et al., 2019).
- Each chip on the wafer is tested for correct performance, and then separated from other chips on the wafer by a saw. In view of this, good chips are placed in supporting packages that allow them to be plugged into circuit boards and bad chips are marked and discarded. In short, micro fabrication techniques (Heikkinen et al., 2019; Sen et al., 2019) have been contributed immensely to molecular technology, cell biology and medicine. The fabrication of a controlled drug delivery vehicle concerning architecture, topography, and functionality results in high predictability of in vitro and in vivo. So, these microchips represent as one of the most advanced fabricated well-developed technology and capable of releasing the drug over a longer period. The first microchip was developed in the year 1999 and this technology should be achieved already in vitro and in vivo with selected therapeutic agent along with regular pulses of drug expulsion into the experimental system (Haitao et al, 2019; Kmiec et al., 2019). Most of the therapies given to the patient which requires that particular type of drug should be repeatedly administered or in specific amounts at a time to maximize drug effectiveness (Haitao et al, 2019; Kmiec et al., 2019). However, various applications are applied in various, or different fields of medicine as shown below-
- This microchip based technique is applied in cancer therapy, especially for measuring the concentration of proteins within the blood (Hui et al., 2018; Yao et al., 2019). So doctors monitor the patients' health status especially in chronic diseases. Now a day, current methods for testing these blood proteins are too expensive and require too much blood to be performed regularly. In view of this, micro fluidic

chip is applied especially in clinical trials, and normally does on a single chip and results should come within 10 minutes. Similar types of results obtained by technician but it takes several hours to do this process only single drop of blood. So, this microchip based technology should give some hope and can be tried to use in diagnostic purpose. As per the literature, first development of microchip chip was reported by Caltech chemistry professors, James Heath and Leroy Hood, President and founder of the Institute for Systems Biology, in Seattle. Finally, Heath and Hood, formed a company called as Integrated Diagnostics pertaining to commercialize the blood chip (Irimia and Wang, 2018).

- This microchip based technology is applied in medical sciences, especially by doctors. These microchips are directly embedded in the patients body and doctors should monitor as well as control the drug release into their patients' body remotely through wireless connections, (Farra et al., 2012). One of the examples is seen in women where microchip drugs were given as hormones for bone strengthening, because they were suffering from osteoporosis and required daily injections of these hormones. According to the reports, this microchip was removed from the patient body after four months (Farra et al., 2012).
- Collaboration between Bio microelectromechanical Systems (BioMEMS) Resource Center and Massachusetts General Hospital (MGH) Cancer Centre has developed a microchip-based device pertaining to isolation, enumeration and analysing circulating tumor cells (CTCs; viable cells from solid tumors) from a blood sample. In this regard, CTC chip is prepared and showing some potential and considered them as valuable tool for monitoring as well as guiding the cancer treatment process (Haitao et al, 2019).
- The development of a microchip, especially for tuberculosis (TB) ELISA, is mainly responsible for detecting IgG responses against multiple type of antigens from plasma samples of active TB (ATB) patients in a rapid, and miniaturized detection system. This microchip utilizes Mycobacterium tuberculosis, surface glycolipid i.e. trehalose 6,6′-dimycolate (TDM) and two purified proteins, 38 kDa glycolipoprotein and antigen 85A (Ag85A), as antigens based on their known immunogenicity and their application in TB serodiagnosis (Gijs, 2004; Hua et al, 2019; Schneider et al., 2019).
- Both companies i.e. Massachusetts Institute of Technology, and Case Western Reserve University, developed microchip which holds measured doses of teriparatide (i.e. Forteo), injectable drug and is generally used to treat osteoporosis. This drug is difficult to administer but daily injections are required. In an effort to overcome this problem, chip (assembly consists of two chips on the surface of a titanium housing that holds the electronics) may be implanted in women who are suffering from osteoporosis but are healthy. As per the reports given by the doctors it was a surprise, that drugs dispensed through microchips showed more effective results as compared to those who had received ordinary injections as there was an increase in bone mass and mineral density as well but with no serious side effects (Gijs, 2004; Hua et al, 2019; Schneider et al., 2019).
- One of the approved FDA chips named as Verichip (now called Positive ID i.e. radio-frequency identification, RFID) started in the year 2004, allowed the physicians to easily access their patients, especially their health records. The size of the chip is equivalent to the size of a rice grain and actually can be implanted in the patient's arm. Instead, it contained a unique 16-digit identification number that would appear when the chip was scanned. With the help of microchip identification number, medical staff could easily access the patient's health records, (Gijs, 2004; Hua et al, 2019; Schneider et al., 2019).
- This chip technology is applied in diagnosis of various infectious diseases, especially detection of disease microorganisms (causing malaria, tuberculosis, diarrhoea, pertussis, and dengue); enteric infections (Escherichia coli O157:H7, Shigella dysenteriae, Salmonella, and Shiga Toxin-producing Escherichia coli). These organisms can be detected from a small amount of fecal sample on a microchip. In addition, microchips, awarded AAPS Drug Delivery Technology Award in the year 2008 for outstanding research in osteoporosis (Gijs, 2004; Hua et al, 2019; Schneider et al., 2019).

CONCLUSION

In medical science, chip based technology should transform or help conversion from unsustainable type of healthcare systems into more sustainable ones, which may equalize the relationship of medical professionals, doctors, scientists and patients. As this technology may provide cheaper, quicker and more effective means against various diseases it may help us win the battle for us against various dreadful pathogens. In short, microchip based technology is helpful in diagnosis as well as the screening of diseases, and also can be applied to other applications related to human health as well. Apart from these, this technology has played an important role in areas of drug discovery and delivery with respect to synthesis, screening of pure compounds, undergoing preclinical testing in vitro and in vivo. Recent developments and applications of microchip based devices have established fool proof research methods for analysing the effects of drugs at different time intervals as well. The ability of these microchips should be further used at the micro or nano level, which can be applied in high precision drug research including toxicity and pharmacokinetics.

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Antitumor L-Glutaminase Production by Rare Actinomycetes Obtained from Marine Water Using Submerged Fermentation

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ABSTRACT

Rare actinobacteria have received significant attention which are commonly categorized as strains other than Streptomyces or isolates with less frequency of isolation under normal parameters. These rare actinomycetes may produce important enzymes and some antibiotics. L-glutaminase is an amidohydrolase, produced by a variety of microorganisms including bacteria, yeast and fungi and catalyzed the deamination of L-glutamine to glutamic acid and ammonia. It has extensive applications as an antitumor agent. About 11 actinomycetes isolates were obtained from saline water from the Red Sea Coast on starch nitrate agar containing 10 % NaCl and some antibiotics (25 µg /ml nystatin, 25 µg/ml novobiocin and 25 µg/ml cycloheximide. at 45°C. All the isolates were screened for L- glutaminase production using phenol red as indicator. Out of 11 isolates, 5 showed excellent production and the isolate MM11 was the most active one. According to morphological and physiological characters, it was identified as identified as species of genus Streptomyces. Identification was confirmed using 16SrDNA and the isolate was identified as Streptomyces sp. MM11 and was similar to *Streptomyces barkulensis* strain RC1831 with 95% similarity level. Thus, it was identified as *Streptomyces barkulensis* MM11. Maximal enzyme production was detected in medium containing L-glutamine as carbon and nitrogen sources, respectively at pH 9.0, 40°C and after 7 days. It was clear that addition of yeast extract decreased the enzyme production. The enzyme was collected; partially purified using column chromatography. The molecular weight was determined to be 44 kD. Brine shrimp lethality test was used to predict the cytotoxic effect of the L-glutaminase. The obtained enzyme showed no toxicity and excellent antitumor activities against two cancer cell lines. In conclusion, using submerged fermentation, L-glutaminase was produced by *Streptomyces barkulensis* MM 11 using maltose and glutamine as carbon and nitrogen sources and optimizing the growth conditions enhanced the enzyme production which can be used as antitumor agent with no toxicity.

KEY WORDS: L-GLUTAMINASE, ANTITUMOR, TOXICITY, STREPTOMYCES, 16SRRNA, SCREEN, ISOLATION, ANTIBIOTIC.

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INTRODUCTION

Actinomycetes are a heterogeneous group of Gram-positive bacteria with high G+C content in their DNA >55%. They are one of the most diverse groups of filamentous or non-filamentous bacteria, among which some genera produce spores and exhibit powdery growth (Laycock et al., 2013 Takahashi and Nakashima, 2018). The phylum actinobacteria represents one of the largest among the 30 major phyla within domain Bacteria. There are 6 classes, 18 orders, 14 suborders, 63 families and 374 genera were recorded (Subramani and Aalbersberg, 2012). The genus Streptomyces belongs to actinomycetes and contains the largest number, about 600 species (Han et al., 2015). It has an enormous biosynthetic potential that remains unchallenged without a potential competitor among other microbial groups (Solanki et al., 2008).

Rare actinobacteria are commonly categorized as strains other than Streptomyces or actinobacterial strains with less frequency of isolation under normal parameters ((Berdy, 2005, Baltz, 2006). Isolation of actinomycetes for novel compounds from conventional habitats had led to rediscovery of known compounds. However, there is still an urgent need for discovering novel secondary metabolites to combat the problem of arising number of resistant pathogenic bacteria and increasing need of more efficient enzymes in pharmaceutical industries (Rangseekaew and Pathomaree, 2019).

Consequently, the search for novel products has switched to rare genera of actinomycetes from normal habitats or to discovery of strains/species found in unusual or unexplored habitats. Almost every group of organisms isolated from marine environment has unique structures. New and rare actinomycetes have been isolated from the marine environment. Rare actinomycetes may be a source of L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) which is a hydrolytic enzyme that catalyzes the deamination of L-glutamine to glutamic acid and ammonia. L-glutaminase has received significant attention with respect to its extensive applications in pharmaceuticals as an anti-leukemic agent and in food industry as a flavor enhancer (Nakadai and Nasuno, 1989, Nandakumar et al., 2003, Dhevagi et al., 2017 Subramani and Sipkema, 2019).

Another great application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian (Balagurunathan et al., 2010). L-glutaminases are widely distributed in bacteria, yeast and fungi (Nandakumar et al., 2003). L-glutaminases production have been reported from E. coli), *Bacillus subtilis* (Dubey et al., 2015), *Proteus morganni, Proteus vulgaris, Xanthmonas juglandis, Erwnia carotovora, Erwnia aroideae, Serratia marcescens, Enterobacter coacae, Klebsiella aerogenes* and *Aerobacter aerogenes* (Wade et al., 1971). Also, L-glutaminase synthesis has been reported from *Streptomyces rimosus* (Keerthi et al., 1999), *Streptomyces sp.*-SBU1 (Krishnakumar et al., 2011) and *Streptomyces avermitilis* (Abdallah et al., 2013). L-glutaminase was isolated from *Penicillium crustosum, Emericella nidulans* and *Mucor circinelloides*, grown on the selective medium and the effects of pH, L-glutamine concentrations, temperatures, and incubation periods on glutaminase production was studied. They recorded maximum production at pH 8, temperature 30 °C, with 0.6% of the L- glutamine. The enzyme was extracted and purified with gel filtration and ion exchange (DEAE Sephadex A50). The molecular weight has 70 kDa and showed cytotoxic activities against two cell lines (LD50: 0.067 - 0.079 mg/ml), (Khalil et al., 2020).

Recently, Masisi et al. (2020) studied the role of glutaminase in cancer, primarily focusing on breast cancer and they address the role played by oncogenes and tumour suppressor genes in regulating glutaminase. They also discussed the current therapeutic approaches to targeting glutaminase. L-glutaminase productions from microbial sources are become urgent need to over produce the enzyme with new and novel character (Prabhu and Chandrasekaran, 1997). The present study reported the production, purification and characterization of extracellular glutaminase enzyme for biotechnological applications from one of the rare actinomycetes.

MATERIAL AND METHODS

Samples collections for rare actinomycete isolation: Marine water samples were collected from Red Sea Cost in Jeddah city, western region, Saudi Arabia. The collected samples were taken to laboratory in sterile plastic bags and stored at 4^0Cuntil used. From each sample, 0.1 ml was spread on each Petri dish plate containing Starch nitrate medium prepared with 10 % NaCl and containing 25 µg /ml nystatin, 25 µg/ml novobiocin and 25 µg/ml cycloheximide. All plates were incubated at 45°C for 5 days. The colonies which showed powdery growth were selected and transferred to slants of the same medium and preserved at 4°C. For long preservation (more than six months), strains were kept in 20% glycerol and stored at -80°C for further study (Aly et al., 2015).

Screening and selection of L-glutaminase producing isolates: The strains were preliminary tested for L-glutaminase production by streaking on minimal glutamine agar medium (MGA) plates, containing (g/l): KCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; $FeSO_4.7H_2O$, 0.1; $ZnSO_4.7H_2O$, 1.0; glutamine 5 as the sole carbon and nitrogen source and phenol red 0.012 as a pH indicator. All plates incubated at 45°C for 5 days. Formation of pink zones around the microbial growth indicated the positive reaction (Balagurunathan et al., 2010, Balagurunathan and Subramanian, 1993).Secondary screening for L-glutaminase production in liquid medium was carried out by inoculating the strains that showed positive result in rapid screening, in medium containing (g/l): L-glutamine, 20; yeast extract, 0.5; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.1; NaCl, 1.0 (Wakayama et al., 2005). After growth, the clear supernatant was used as crude enzyme (Dura et al., 2002). L-glutaminase production was measured according L-glutaminase assay method described by (Imada et al.,1973). One international unit

of L-glutaminase was defined as the amount of enzyme that liberates one µMol of ammonia under optimum conditions. The enzyme yield was expressed as Units/ml (U/ml).

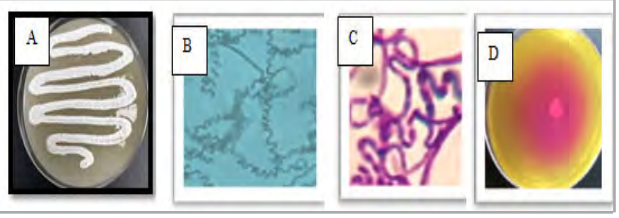
Identification of the bacterial isolate: The bacterial isolate that showing the highest L-glutaminase production was identified using morphological, physiological, biochemical and molecular studies. Molecular characterization was determined after extraction of DNA (Kumar et al., 2010). PCR amplification of the 16S rDNA of the Streptomyces sp. was performed using two primers: 9F (5'-GAGTTTGATCCTGGCTCAG- 3') and 1541R (5'-AAGGAGGTGATCCAACC-3') as recommended by Hall et al. (1999).

L-glutaminase assay: Imada et al. (1973) was used for L-glutaminase assay and the mixture was incubated at 37°C for 30 min. The addition of 0.5 ml of 1.5 M trichloroacetic acid was used to stop the reaction. Enzyme activity was determined (U/ml). One unit of L-glutaminase is the amount of the enzyme that produced a µMol of ammonia.

Effect of growth factors on L-glutaminase production: Impact of different parameters, temperature, pH value, and yeast extract and incubation period on L-glutaminase production by Streptomyces barkulensis was investigated (Aly et al., 2017).

Purification of the L-glutaminase: After precipitation with 80% NH_4SO_4, the enzyme was purified using Sephadex G100 column chromatography and DEAE-Cellulose column chromatography. The active peak was collected, lyophilized and enzyme molecular weight was detected (Aly et al., 2017).

Figure 1: The selected isolate MM 11 on starch nitrate agar containing three antibiotic (A), on slide agar (B), stained with Gram stain (C) and screened for L-glutaminase using phenol red as indicator (D)



Toxicity and antitumor activity:The brine shrimp lethality test was used to predict the cytotoxic effect of the natural products (Meyer et al., 1982). Varying concentrations of L-glutaminase was added to sea water, containing a counted number of live brine shrimp larvae. Control brine shrimp larvae were incubated in a mixture of sea water. After 24hr., the average number of larvae that survived in each vial was determined. The mean mortality level was plotted against the logarithm of concentrations, the concentration killing fifty percent of the larvae (LC_{50}) was determined (Meyer et al., 1982). Similarly, the antitumor activity against Ehrlich carcinoma and Lymphoma cell line were determined. The cells were grown in RPMI 1640 medium (Sigma, USA) with 10% fetal calf serum (Gibco, USA) at 37°C under a humidified atmosphere consisting of 95% air and 5% CO_2 for 48 hr. Cells were treated with different doses of the L-glutaminase for 24 hours, centrifuged for 2 min at 1500 g and counted using hemacytometer after staining with trypan blue and removing the supernatant. The percentage of cell viability was assessed to determine the 50 % lethal dose by which 50% of cells are killed (LD_{50}) (Al-Footy et al.,2016).

RESULTS AND DISCUSSION

About 11 actinomycetes isolates were obtained from saline water of the Red Sea Coast in Jeddah city. The isolation medium was starch nitrate agar supplemented with 10% NaCl and 25 µg/ml nystatin, 25 µg/ml novobiocin and 25 µg/ml cycloheximide. The incubation temperature was 45°C. All the isolates were screened for L- glutaminase production using.

Figure 2: The phylogenetic tree of the isolate MM11 and the most related genera

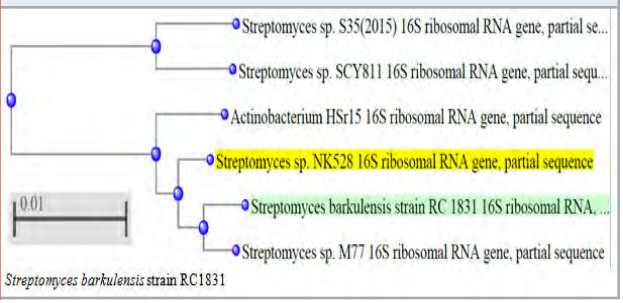
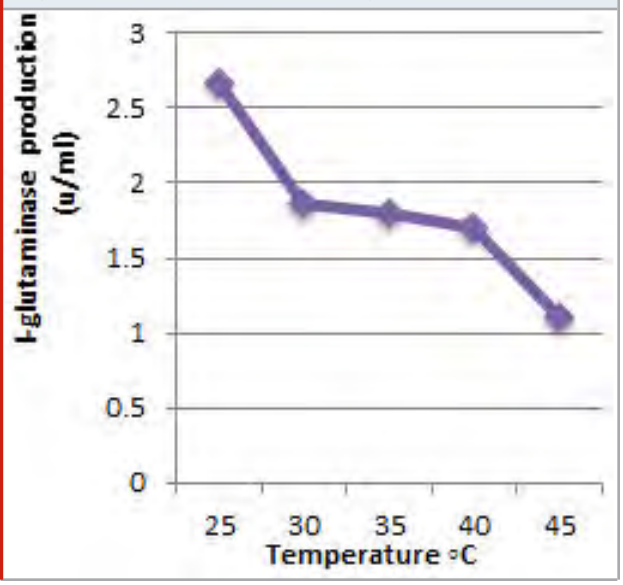


Figure 3: Effect of the temperature on production of L-glutaminase in broth medium by the isolate MM11



L-glutamine as carbon and nitrogen sources. Phenol red was added to the medium as indicator. Out of 11 isolates, 5 bacterial isolate showed the maximum growth and the diameter of the pink zone was ranged from 22 to 37 mm

(Table 1). The most active isolates were grown in liquid medium containing L-glutamine for 5 day. The isolate MM11 was the most active isolate for L-glutaminase production (Figure 1). The selected isolate was grown on different agar media, growth, color and pigment production were recorded (Table 2). Moreover, some physiological characters and resistant to some antibiotics were determined (Table 3). The growth on different carbon and nitrogen sources were reported in Table 4.

Figure 4: Effect of the pH value on production of L-glutaminase in broth medium by the isolate MM11

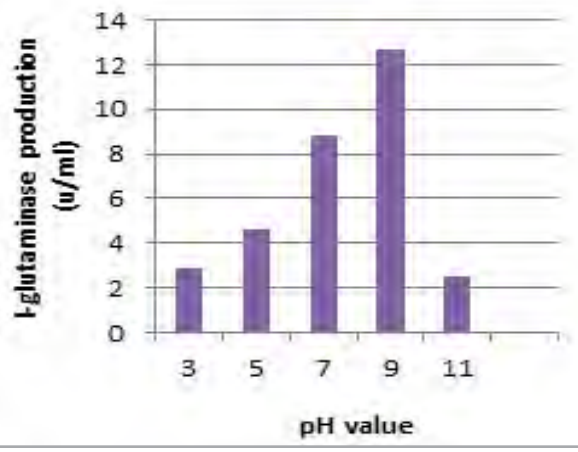


Figure 5: ffect of the yeast extract addition on production of L-glutaminase in broth medium by the isolate MM11

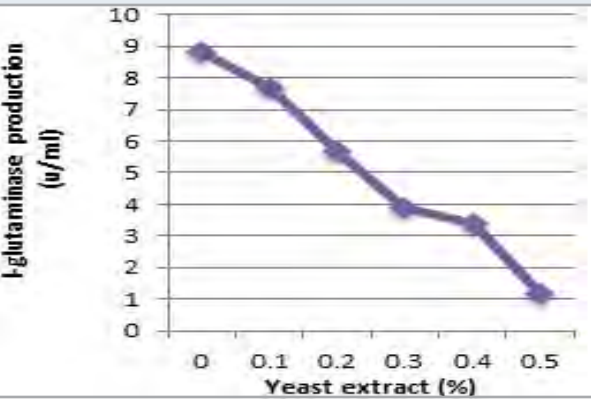


Figure 6: Effect of the incubation period on the production of L-glutaminase in broth medium by the isolate MM11

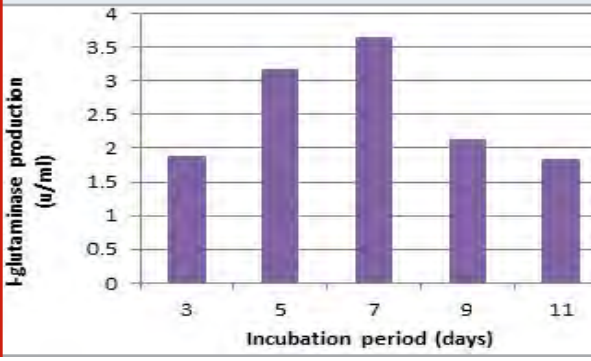


Figure 7: Elution profile of L-glutaminase of the isolate MM11 after sephadex G-100 column chromatography (A) and DEAE-Cellulose column chromatography (B).

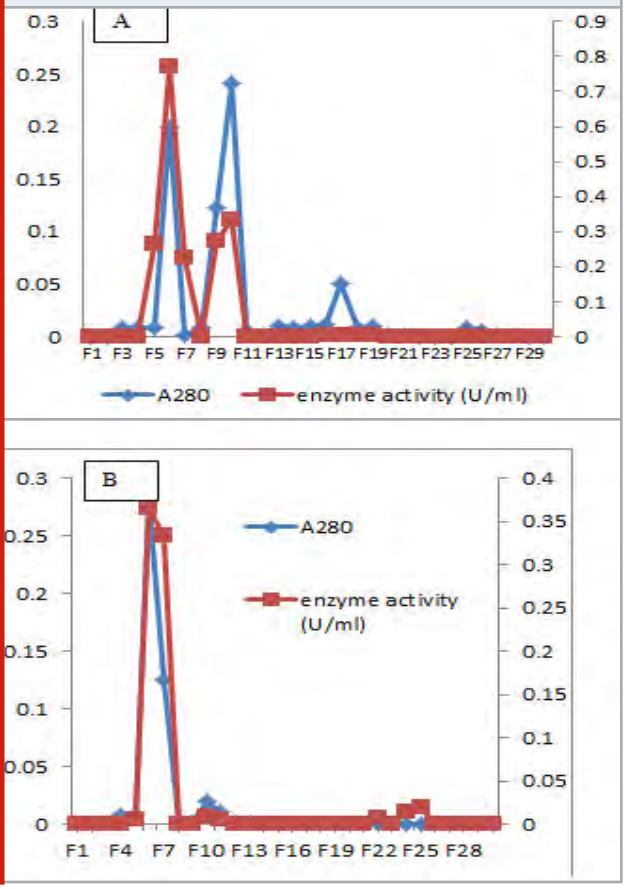
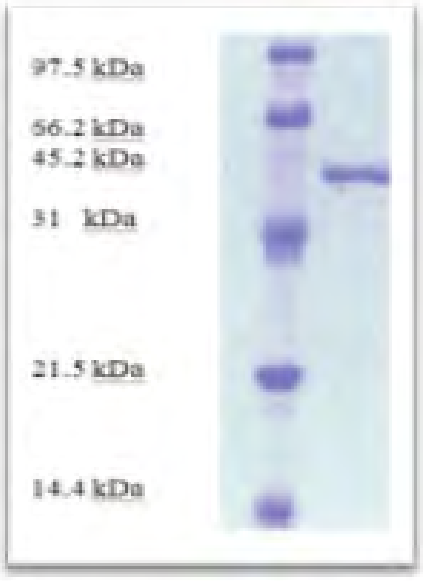


Figure 8: SDS-PAGE analysis of the purified enzymes after DEAE-cellulose column chromatography



According to morphological and physiological characters, the most active isolate was identified as species belong to genus *Streptomyces*. Identification was confirmed using 16SrRNA and the isolate was identified as S*treptomyces barkulensis* (Figure 2). Maximum L-glutaminase production was detected in broth medium contained L- glutamine as carbon and nitrogen sources at 45°C, pH 9.0, no yeast extract addition and after 7 days (Figures 3, 4, 5 and 6). Addition of yeast extracts decreased L-glutaminase production. In broth medium, the selected bacterium was grown in Lab. scale production, the enzyme was collected and purified. After precipitation with 80% NH4SO4 the crude enzyme was partially purified using column chromatography. Molecular weight of the purified L-glutaminase was determined using gel electrophoresis. The molecular weight was of the pyre enzyme was determined to be 44 kDa (Figure 8). Brine shrimp lethality test was used to predict the cytotoxic effect of the L-glutaminase. The obtained enzyme showed no toxicity and excellent antitumor activities against two cancer cell line (Table 5).

Table 1. Source of some bacterial isolates from marine water and their L-Glutaminase production on minimal agar medium (Pink zone diameter/ mm) and in liquid broth medium (u/ml).

BACTERIAL ISOLATE	SOURCE	COLOR OF THE ISOLATE	GROWTH ON AGAR MEDIUM	L- GLUTAMINASE DETECTION	
				ON SOLID AGAR (DIAMETER OF PINK ZONE, MM)	IN LIQUID BROTH MEDIUM (U/ML)
MM1	MARINE WATER	BLUE	+++	33±3.31	12.10 ±2.19
MM5	MARINE WATER	DARK GRAY	+++	25±0.94	6.68 ±1.39
MM9	MARINE WATER	BLUE	+++	24±5.31	10.01±1.22
MM11	MARINE WATER	YELLOWISH WHITE	+++	37±3.39	18.14 ±2.04
MM13	MARINE WATER	DARK GRAY	+++	22±0.39	6.13 ±1.34
LSD 6.66					

Table 2. The selected actinomycetes MM11 on different media after growth for 5 days at 30°C.

MEDIA USED	GROWTH	COLOR OF AERIAL MYCELIUM	COLOR OF SUBSTRATE MYCELIUM	PRESENCE OF SOLUBLE PIGMENT
STARCH- NITRATE AGAR	HEAVY	PALE YELLOW	YELLOW	NO PIGMENT
YEAST EXTRACT- MALT EXTRACT AGAR (ISP-2)	HEAVY	WHITE	YELLOWISH WHITE	NO PIGMENT
IN-ORGANIC SALTS-STARCH IRON AGAR (ISP-4)	POOR	WHITE	WHITE	NO PIGMENT
GLYCEROL ASPARAGINE AGAR (ISP-5)	HEAVY	WHITE	WHITE	NO PIGMENT
TYROSINE AGAR (ISP-6)	MODERATE	WHITE	YELLOWISH WHITE	NO PIGMENT
E-MEDIUM (ISP-9)	POOR	WHITE	WHITE	NO PIGMENT

Table 3. Antibiotic susceptibility and physiological and biochemical tests of the selected isolate MM11

TESTS	RESULT	TESTS	RESULT
GELATINE PRODUCTION	_	AMIKACIN	SENSITIVE
MELANIN PRODUCTION	_	CEFTAZIDIME	RESISTANT
STARCH HYDROLYSIS	+	AZTREONAM	RESISTANT
CATALASE	+	PIPERACILLIN	RESISTANT
OXIDES	+	IMIPENEM	SENSITIVE
INDOLE TEST	+	CIPROFLOXACIN	SENSITIVE
METHYL RED TEST	+	AMPICILLIN	SENSITIVE

The chance of isolating new actinomycete strains with novel character is difficult and need special methods to be isolated. Addition of NaCl to the isolation medium enhanced salt tolerant actinomycetes. Similarly, addition of antibiotics to the growth medium enhanced rare actinomycete isolation. Selective isolation of actinomycetes for L-glutaminase production is of great interest for preparing new antitumor agents. Out of 11 actinomycetes isolates, 5 isolates (45.5%) were highly producer of L-glutaminase and the most active isolate was *Streptomyces barkulensis* Screening was performed based on the activity of glutaminase (33 u/ml). Similarly, Out of 102 actinomycete isolates, only 6 Streptomyces isolates recorded L-glutaminase activities (Abdallah et al., 2012).

Detection of L-glutaminase in this study was recorded by diameter of the pink zone (mm) and the same method was used (Abdallah, et al., 2012). Similarly, L-glutaminase was obtained by *Streptomyces avermitilis* (Omura et al., 2001) and Streptomyces labedae (Han et al., 2012). Effect of various physicochemical factors on L- glutaminase production by *Streptomyces species* was detected. It was found that under the best growth conditions, rapid L-glutaminase production was found (Tobin et al., 2001). On the other hand, there is a dire need for the discovery of new drugs to effectively target the life-threatening diseases like cancers. The application of enzymes in diverse biotechnological industries tends to the discovery of novel enzymes. Members of the class actinobacteria especially Streptomyces spp. have long been recognized as prolific sources of useful bioactive enzyme like

Table 4. Effect of different carbon and nitrogen sources in ISP-9 medium on growth of the selected isolate MM11.

CARBON SOURCE	UTILIZATION	NITROGEN SOURCE	UTILIZATION
NEGATIVE CONTROL	++	AMMONIUM SULFATE	+
POSITIVE CONTROL (GLUCOSE)	+++	AMMONIUM	
CHLORIDE	+		
SUCROSE	++	SODIUM NITRATE	++
STARCH	+++	POTASSIUM NITRATE	+
LACTOSE	+++	GLYCINE	++
DEXTROSE	-	PEPTONE	+++
MALTOSE	+	YEAST EXTRACT	+++
GLYCEROL	+	VANILLIN	+++
XYLOSE	-	ASPARAGINE	+++

+++: Good utilization, ++: Moderate utilization, +: Poor utilization, -: No utilization

Table 5. Toxicity (mg/ml) against *Artimia salina* and the antitumor activities (LD50, mg/ml) of l-glutaminase.

Tested Product	Toxicity against Artimia salin (LC_{50})	Antitumor activity (LD_{50})	
		Lymphoma cell line	Erlish cell line
Filtrate	≥77.0*	≥77.0*	≥77.0*
L-glutamimase	≥55.3*	5	7.5
Control (cis platin)	≥ 3.0	3.6	≥ 3.6

* : significant results compared to contro

L-glutaminase (Nathiya et al., 2011). Therefore, current actinomycetes isolation programs are reoriented toward largely unexplored and extreme environments. The present study has been focused on the isolation and identification of different actinomycetes from extreme habitats in Saudi Arabia. Marine environments were studied in order to unravel the diversity of actinomycetes and determine their potential as a resource for biotechnological applications. The findings obtained from this study revealed that the most active isolate was of genus *Streptomyces* (Nonomura, 1974).

L-Glutaminase, an amidohydrolase enzyme has been a choice of interest in the treatment of lymphoblastic leukaemia. The isolate was identified as Streptomyces sp. Effect of physicochemical factors namely temperature, pH, yeast extract concentration, and incubation period on the production of L-glutaminase from the *Streptomyces* was carried out. The enzyme production was found to be optimum in medium containing L-glutamine as carbon and nitrogen source at pH 9, temperature 45^{0}C The L-glutaminase produced from *Streptomyces sp.* was purified by ammonium sulphate precipitation, dialysis method and ion exchange chromatography.

After the purification of the enzyme by ion exchange chromatography, it has 44 kDa. Similar results were obtained by (Okami (1986), Omura et al., 2001).

CONCLUSION

Rare actinomycetes were found in extreme environments and need special method to be isolated. *Streptomyces barkulensis* isolated from marine water in medium containing three different antibiotics. It was characterized and identified. The previous isolate showed excellent activity of L-glutaminase production. Optimization of growth conditions enhanced the production process. Using Lab. scale production the enzyme was purified and characterized. It showed no toxicity and moderate antitumor activity. Thus, it can be developed for medical uses.

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Improving the Level of Socio-Psychological Adaptation in First-Year Students of a Russian University Moscow, Russia

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ABSTRACT

The adaptation process is always very complicated and is determined by the current level of health that minimizes the risk of developing diseases. The study of the adaptive capabilities of a particularly young organism for this reason should be comprehensive and necessarily have a psychological component. The admission of young people to higher education is a serious stress for her. Studying at the university is also a stress, requiring serious strain of various adaptive mechanisms, which affects the physical and psychological status of students. Of particular interest is the adaptation of first-year students to study at the university, which is a complex socio-psychophysiological stimulus for all body systems. Various approaches to the elimination of cognitive distortions are of great importance for optimizing adaptation in psychotherapy. Desensitization with the processing of psychological injuries with eye movements has proven itself to be a highly effective option for psychotherapy. The use of the author's version of such psychotherapy provided a correction for first-year students in the level of socio-psychological adaptation by increasing their psychological age and level of personal self-esteem, willpower while attenuating manifestations of sociophobia.

KEY WORDS: STUDENTS, PSYCHOLOGICAL ADAPTATION, EYE MOVEMENT DESENSITIZATION AND REPROCESSING, PSYCHOLOGICAL AGE, WILLPOWER, SOCIOPHOBIA.

INTRODUCTION

The adaptation process reflects many facets of the interaction of the body with the environment (Bespalov et al., 2018a), is determined by the current level of health (Makhov, Medvedev, 2020a) and the risk of developing diseases (Karpov et al, 2020). The study of the adaptive capabilities of a particularly young organism for this reason should be comprehensive (Zavalishina, 2018a; Zavalishina, 2018b). A serious stressful environmental impact for young people is admission to a higher educational institution (Makhov and Medvedev, 2018a). The subsequent study at the university also requires seriously straining various adaptive mechanisms. This inevitably affects the physical and psychological status of students, causing significant stress on all body systems (Andrienko et al., 2019).

The success of the adaptation of students in the first year to a large extent determines the overall effectiveness

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of their education (Bespalov et al., 2018b). Difficulties with adequate adaptation to changing conditions of the social environment are manifested by the low level of their socio-psychological adaptation, which prevents the formation of professional competencies in them (Makhov and Medvedev, 2018b). Various sociophobia, the low level of their psychological age, and reduced self-esteem of the strength of volitional processes can seriously hinder the development of social adaptation of students (Bespalov et al., 2018c).Various approaches to eliminating cognitive distortions that complicate the process of socio-psychological adaptation of a person have great effectiveness in optimizing the socio-psychological status and ensuring adaptation in psychotherapy (Makhov and Medvedev, 2020b).

A very effective variant of psychotherapy proven desensitization reprocessing psychological trauma eye movements. This method is considered very effective and successfully applied to recover from emotional shocks. It is used to minimize the manifestation of post-traumatic syndrome, the syndrome of dependence or of depression caused by bereavement (Biserova and Shagivaleeva, 2019). This methodology promotes synchronization of rhythms in the cerebral hemispheres, provides the optimum activated, and provides simultaneous information processing (Cuijpers et al., 2020). It can be applied at any age of a person in a stressful situation. In this regard, the authors felt justified to test the effectiveness of the author's method of desensitization reprocessing psychological trauma eye movements to increase the level of social adaptation of young people who began teaching in higher education. The objective was to evaluate the effectiveness of the author's method of processing psychological trauma to the eye movements relative to the correction of socio-psychological adaptation of first-year students.

MATERIAL AND METHODS

This study was supported by a meeting of the local ethics committee created at the Russian State Social University on September 15, 2018 (protocol №11). The study was taken by first-year students of the Russian State Social University (Moscow, Russia) with a total of 56 people, average age 22.1±0.52 years, including 9 boys and 47 girls. Examination students did not have bad habits, any mental disorders and chronic somatic diseases before taking into the study and throughout the observation.

To assess the dynamics of students' adaptation to study at the university, all those taken into the study were randomly divided into two equal, comparable, homogeneous groups-the experimental (n=28) and control (n=28). In the experimental group, the author's methodology was used to increase the level of socio-psychological adaptation at the university. In the control group, the process of adaptation of the observables went naturally without outside interference. The entire study was conducted over one semester (4 months). Testing was carried out in both groups simultaneously: initially and after 4 months of exposure in the experimental group (Zavalishina, 2018a).

The author's technique applied in the experimental group included the following components. Students were shown 32 copyright videos on a widescreen screen with their display at eye level. The duration of each demonstration was 10-12 minutes. The videos contained materials on 32 topics. The demonstrated material contained forms of human behavior that were approved in society (marriage, parenting, saving animals in natural disasters, harvesting in severe weather conditions, and so on), a direction to increase mental health.

Before each viewing of the video material, the subjects were asked to immerse themselves in the memories of unpleasant moments from their study at the university, which they would once cause psychological trauma and rate on a scale from 0 to 10 (where "0" is complete indifference, and "10" is the maximum possible intense experience) how much it bothers them. In this case, the subjects had to remember what feelings they experienced at that moment, to remember the words or sounds that accompanied them at the time of the formation of these experiences.

After that, a video was included, where the main elements that captured the subjects' attention rhythmically and systematically moved around the screen, producing a cognitive effect in the form of an information load throughout the viewing. At the end of each viewing, the subjects had a conversation about how they would now behave in a traumatic situation and how their feelings for psycho-traumatic situations that were remembered before watching changed. The question was asked - will similar situations affect their mood and emotional background in the future? A number of tests were used to assess the dynamics of the state of students under observation.

1. Test "What are we afraid of." This test contained 60 statements, which are a free statement of basic human fears. Using this test, we determined the general level of social fear and its level in any sphere of life with the differentiation of fear into a conscious and unconscious component (Nekrasov, 2018). With the help of the applied test, sociophobia was diagnosed when the results reached 9 points or more.
2. The test "Self-esteem of willpower" made it possible to determine the degree of assessment of the manifestation of one's own "willpower" (Ilyin, 2009). The results obtained using this test were evaluated based on the following criteria:

from 1 to 10 points-low motivation for success;
from 11 to 16 points-the average level of motivation for success;
from 17 to 20 points-a moderately high level of motivation for success;
over 21 points-too high level of motivation for success.
3. Test for psychological age. Its use made it possible

to evaluate the self-awareness of one's psychological age and tone, as well as the degree of psychological maturity of the subject (Stepanov, 2000). The values of the results of the application of this test were expressed in years, making it possible to establish deviations of the obtained data from the biological age of the subject. Mathematical processing of the digital material obtained in the study was carried out by a standard package of statistical programs using Student t-test, which allows to find the reliability of differences between the level of compared indicators.

RESULTS AND DISCUSSION

The data obtained during the study are presented in table 1.

Table 1. Dynamics of indicators of socio-psychological adaptation in the examined students

Parameters	Experienced group, M±m		Control group, M±m	
	at the beginning of the study, n=28	at the end of the study, n=28	at the beginning of the study, n=28	at the end of the study, n=28
Sociophobia level, points	9.2±0.01 p<0.01	2.7±0.20	9.1±0.08	8.6±0.11 p_1<0.01
Self-will, points	12.0±0.75	15.0±0.87 p<0.01	10.0±0.93	11.0±0.97 p<0.05 p_1<0.01
The level of psychological age, years	19.3±0.65	23.4±0.68 p<0.01	18.9±0.38	19.3±0.54 p_1<0.01

Legend: p – is the reliability of the dynamics in the groups, p1– is the reliability of the differences of the surveys at the end of the observation between the groups.

Initially, the performance of both groups of subjects did not have statistically significant differences. In both groups, at the beginning of the observation, there was sociophobia, low self-esteem, and the level of psychological age was inferior to the calendar.

As a result of applying the author's methodology in the experimental group, it was possible to increase the level of socio-psychological adaptation and achieve significant positive changes in the recorded parameters. By the end of the observation, the experimental group experienced a 3.4-fold decrease in the level of sociophobia, increased self-esteem of willpower by 25.0% and an increase in the level of psychological age of the examined people by 21.2%, with its reaching the calendar level. The natural course of the processes of socio-psychological adaptation in the control group was accompanied by a weak dynamics of the recorded indicators

By the end of the observation in the control group, the level of sociophobia decreased by only 5.8% and was 3.2 times lower than the same indicator in the experimental group. By the end of the observation, the self-assessment of willpower in the comparison group increased by 10.0%, yielding 36.4% at the same time in the experimental group. The level of psychological age in students who made up the control group, by the time the observation was completed, increased by only 2.1%, did not reach the calendar age and yielded 21.2% in the experimental group.

An experienced group of students successfully passed their first debt-free session. The average score for the first session of students in this group was 4.3±0.45 points. In the control group, according to the results of the first session, there were 10 debts, and the final average score for these students at their first session at the university was 3.8±0.25 points.

Recently comes a clear understanding of the need to identify people have traumatic experiences, are able to form their dysfunctional behavioral patterns and symptoms of social and psychological disadaptation (Makhov and Medvedev, 2018c; Skoryatina and Medvedev, 2019). They degrade their interaction in society in General and especially in their microenvironment (Makhov and Medvedev, 2018d). Early detection and adequate correction of these negative experiences is able to provide quick adaptation of students to training conditions and enhances the success of the assimilation of its educational programs (Nekrasov, 2018).

In this study, it was found that for first-year students tend to have high levels of social anxiety due to low self-assessment of strength of will at the level of mental age below the calendar. Such features obstructs social-psychological adaptation in the learning process at the University and lowered the quality of learning material. Largely at the heart of these phenomena lies the negative information as if "frozen" in the mind and a long time continued in its original, unprocessed form. This is possible due to isolation of the neural networks of the brain that the negative memories from the rest of its associative networks. While in this part of the memory change is not happening, as the information is able to be psycho corrective could not affect isolated information about a traumatic event. In this situation, negative emotions, images, sensations, and views from the past

penetrate the present and cause severe psychological and physical discomfort.

To correct the current situation in students, the Eye Movement Desensitization and Reprocessing method was used, the essence of which was to overcome the consequences of severe mental injuries and stresses that block the activity of the adaptive information processing system in the brain. This method minimizes traumatic memories and related affective, somatovegetative and behavioral reactions that continue to be stored in the brain. This effect occurs due to the activation of eye movements that occur during the implementation of Eye Movement Desensitization and Reprocessing. They start processes that activate the accelerated processing of traumatic experience by analogy with what happens at the stage of sleep with rapid movements of the eyeballs (Makhov and Medvedev, 2018e).

The use of the repeated series of eye movements during the Eye Movement Desensitization and Reprocessing procedure with first-year students led to the unblocking of isolated sections of the neural network of their brain with a traumatic experience. Eye movements during the implementation of the method made it possible to "unlock" this part of the brain and process traumatic information (Perlini et al., 2020). Memories that had a high negative emotional charge using this method were translated into a more neutral form, and the corresponding ideas and beliefs became constructive (Shapiro, 1998).

Applying the author's method, the subjects managed to achieve optimization of all the considered parameters. Gain of the eye movement stimulated the flow of information in the brain of individuals of the experimental group, allowing a more integrative work their bark. In these circumstances, the brain has optimized the processing of information in all its departments (Glagoleva et al., 2018; Podymova et al., 2019). Activities conducted cognitive-behavioural psychotherapy have achieved first-year students a significant increase in the level of General socio-psychological adaptation with the achievement of the optimum values of the studied parameters. Carried out to students of psycho-correction has intensified to have cognitive and behavioral manifestations, "harmonisieren" identity and strengthening its adaptive capacity to situations associated with emotional stress (Savchenko et al., 2016; Makhov and Medvedev, 2019).

The strengthening of awareness of subjects having potential volitional processes have enabled to overcome the difficulties of everyday life and situations of psychological discomfort. Obviously, the result of the correction of the freshmen students have been acceptance of themselves as people with extremely high emotional volitional levels of self-regulation. It appeared from them in the positive dynamics of their reactions to the situation, hazards (real and imaginary). Awareness of these changes helped the students to some a "desensitization" to negative situations in society and strengthen their initial weak points in the system of their response to external factors. To achieve this effect was made possible by the elimination from first-year students of the phenomena of psychological immaturity due to maturation of neural connections in their brain on the background of conducted disturbance.

CONCLUSION

Currently, the Eye Movement Desensitization and Reprocessing method has attracted the increasing attention of psychotherapists. This is due to its high efficiency, providing the possibility of accelerated processing of information, which forms a person's psychological stability. Applying the author's version of such cognitive-behavioral therapy, the study succeeded in correcting the state of self-esteem of willpower, the level of psychological age and significantly lower sociophobia in first-year students. The obtained result indicated the successful optimization of socio-psychological adaptation in first-year students and a sufficiently high effectiveness of the applied impact. This gives grounds for recommending a proven author's method for widespread use in any educational institution to increase the level of adaptation of first-year students and greater efficiency in mastering the curriculum.

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Luciferase Reporter Phage Assay for Anti Tuberculosis Screening: Current Status and Challenges

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ABSTRACT

Tuberculosis (TB) is a contagious airborne disease caused by a bacterium, *Mycobacterium tuberculosis* (MTB). Emergence of multi drug resistant (MDR) and extensively drug resistance (XDR) among *M. tuberculosis* strains urged the situation to discovery novel anti TB antibiotics. Many assays were developed based on the whole cell and target, but each having some limitations. Being a slow grower as well as air borne pathogen, adopting suitable assay for anti TB screening is challenging. Here, we discussed about the employment of Luciferase Reporter Phage (LRP) assay for the screening of wide range of compounds against *M. tuberculosis* strains including drug sensitive and drug resistant strains. Literature articles published between 2006 to March 2020 in reputed journal were collected through searching web of science, pubmed and other sites.

This review focus on the articles published on screening of extracts and compounds from natural products, synthetic compounds and nanoparticles from the year 2006 – March 2020 by using LRP assay. Among the whole cell assays, LRP assay provide the results in 72 hours and this assay can be used as preliminary identification of potential anti-TB compound. Hence, LRP assay is a rapid, simple and sensitive assay to screen natural molecules and synthetic compounds to determine their anti TB activity. However, limitations associated with mycobacteriophage entry into the mycobacterial cell need to be optimized to improve its sensitivity. Understanding the importance and advantages of employing LRP assay as an effective high throughput screening method helps in the significant screening of wide range of antimycobacterial agents in a relatively short time of incubation. This assay can effectively help in the development of new potential drug candidates against tuberculosis..

KEY WORDS: TUBERCULOSIS, LUCIFERASE REPORTER PHAGE ASSAY, ANTI TB SCREENING, DRUG DISCOVERY.

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INTRODUCTION

Tuberculosis (TB) is a communicable disease that is a major cause of ill health, one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent. Globally, an estimated 10.0 million people fell ill with TB in 2018, a number that has been relatively stable in recent years. The burden of disease varies enormously among countries, from fewer than five to more than 500 new cases per 100 000 population per year, with the global average being around 130. There were an estimated 1.2 million TB deaths among HIV-negative people in 2018, and an additional 251 000 deaths among HIV positive people (Global Tuberculosis Report, 2019).

Tuberculosis pose serious problem around the world by the way of increase in the rate of HIV-related TB, pediatric TB, latent TB, MDR- TB and XDR-TB. Drug-resistant TB continues to be a public health threat. In 2018, there were about half a million new cases 5 of rifampicin-resistant TB (of which 78% had multidrug resistant TB). The three countries with the largest share of the global burden were India (27%), China (14%) and the Russian Federation (9%). Globally, 3.4% of new TB cases and 18% of previously treated cases had multidrug resistant TB or rifampicin-resistant TB (MDR/RR-TB), with the highest proportions (>50% in previously treated cases) in countries of the former Soviet Union. The treatment for tuberculosis requires 6-8 months for new cases and 18-24 months for MDR TB with more toxic drugs and the treatment options for XDR-TB are seriously limited.

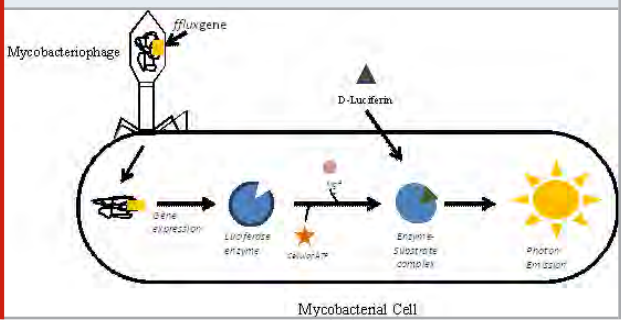
The risk of serious adverse events such as hepatotoxicity, discourage both patients and providers (Menzies et al., 2011). Hence, there is always an urgent need for the development of potential candidate to fight against drug resistant strains with improved activity, novel mechanism, with a short duration for treatment by fast acting mechanism. This necessarily leads to the interest in screening the new compounds for their antimycobacterial activity. Both target based and whole cell screening approaches are in practice for anti TB drug discovery however both of them has its own merits and limitations. Conventional whole-cell screening assays were found to have a higher success rate in identifying a series of hits possessing beneficial properties (Kumar et al., 2017).

It is well known that, *Mycobacterium tuberculosis* is a slow-growing pathogenic organism and its complex cell wall with mycolic acids and other lipid contents, poses numerous restrictions for anti-TB drug research and development (Favrot et al., 2012). Due to the slow growing nature of *M. tuberculosis*, a screening technique based on growth is difficult and new assays for antimycobacterial screening of natural products and synthetic compounds are required (Forbes et al., 2015).

Background and Purpose: Native compounds acquired from microbial resources and medicinal cultivars have played an essential part as the origin of TB medications (Sari et al., 2019). Slow growth rate of the *Mycobacterium* species and their long incubation period remains a major obstacle in the antimycobacterial drug discovery process. Drug susceptibility method using egg or agar is the standard method being used for the evaluation of antimycobacterial agents. However, the assay is labor intensive and the incubation time requires upto 2 months (Rakhmawatie et al., 2019). LRP assay is a high throughput screening method and has been used to evaluate the natural, synthetic and nanoparticles for their antimycobacterial activity (Sivaraj et al., 2020). This assay reveals the potential of native compounds in 3-4 days to behave as an antimycobacterial compound. Understanding the importance and efficiency of LRP assay and application of this assay for the screening process against *M. tuberculosis* strains can help the effective drug discovery process against tuberculosis.

Luciferase Reporter Phage (LRP) Assay: Luciferase reporter phage assay (LRP) assay utilizes genetically modified mycobacteriophages expressing luciferase gene fflux. Mycobacteriophage have been used as diagnostic tools for tuberculosis and also have various applications in mycobacteriology including gene replacement, development of integration proficient vectors, systems for mycobacterial gene expression, mycobacterial cell wall analysis, delivery systems for reporter phages & transposons, etc,(Parikh et al., 2013; Hatfull, 2014; Fu et al., 2015). In principle, when the constructed phage infects the viable mycobacterial cell, the luciferase gene gets expressed upon addition of luciferin substrate which results in emission of light in the presence of cellular ATP and Mg^{2+}. The emitted light is measured using luminometer which displays proton as Relative Light Unit (RLU) (Fig.1).

Figure 1: Luciferase Reporter Phage (LRP) Assay: Genetically modified mycobacteriophages expressing luciferase gene (*fflux gene*) infecting viable mycobacterial cells. The gene gets expressed and upon the addition of substrate luciferin results in emission of measurable light in the presence of cellular ATP and Mg^{2+}. The emitted light is measured in the luminometer.



The first evaluation of the diagnostic LRP assay in sputum samples using phAE142 (mycobacteriophage) was reported by Banaiee et al (2001). The authors found the assay comparable with MGIT 960 in sensitivity, specificity and speed. Similar results were reported by Bardarov et al., (2003) as National Institute for

Research in Tuberculosis (formerly Tuberculosis Research Centre) at Chennai, India, facilitated an ideal, simple and reliable diagnostic LRP assay using phage Che12 and it was found to be the first ever temperate phage capable of infecting and lysogenising *M. tuberculosis*. The specificity of the mycobacteriophages to infect mycobacteria has been effectively put in to use for rapid detection of *M. tuberculosis* in sputum (Kumar et al., 2008). Luciferase phages or *M. tuberculosis* strains expressing luciferase genes may permit rapid screening of drugs for antituberculosis activity.

In this context, LRP assay has been successfully employed for rapid screening of various natural and synthetic and semi-synthetic compounds against *M. tuberculosis* including drug resistant strains and dormant TB bacilli. Limitations associated with mycobacteriophage entry into the mycobacterial cell need to be optimized to improve its sensitivity.The authors have also revealed that this might be achieved by engineering of better characterized mycobacteriophages to allow higher expression of luciferase. The constructed mycobacteriophages such as L5 and D29 have various defects remain in LRP assay especially the mild L5 mycobacteriophage is unable to infect the *M. tuberculosis* complex, which limits its application in the drug resistance *M. tuberculosis* strain detection in the sample (William et al., 1993). In LRP assay, the relative light units can be detected within a few minutes following LRP infection of the live mycobacterial when the sample contains at least 104/milliliter of *M. tuberculosis*. The lytic characteristics of D29 and TM4 result in the loss of light output and reduced sensitivity (Fu et al., 2015).

Kumar et al., (2008) have constructed new LRPs using the mild Che12 bacteriophage to increase light output and improved the sensitivity of the assessment. Temperate phage integrate into the host genome at specific sites and replicate along with cells. Che12, first reported temperate mycobacteriophage capable of infecting and lysogenising *M. tuberculosis* isolated from soil. LRPs developed from temperate mycobacteriophages could be ideal since it does not cells do not lyse, increased light output at a give point could facilitate designing assay format and there is continuous expression of luciferase. Solvents like DMSO make cell membrane more permeable and using it in substrate preparation could ensure its better entry into cells. Setting up the primary liquid culture in dilutions could establish ideal multiplicity of infection and ensure cells are in continuous log phase.

Protocol For Lrp Assay: Briefly, about 350 µl of Middle brook 7H9 broth, 100 µl of *M. tuberculosis* cell suspension (#2 McFarland) and 50 µl of test compound (example: 50 µg/ml; 100 µg/ml; 500µg/ml) was transferred to a 1.8 ml sterile cryovial. The negative control was a cryovial containing 400µl of Middle brook 7H9 broth and 100 µl of *M. tuberculosis*H37Rv cell suspension (#2 McFarland). After 72 hours of incubation at 37oC, about 50 µl of high titre mycobacteriophage (titre-6.5x109pfu/ml, the phage was kindly gifted by Dr. Vanaja Kumar, NIRT, Chennai)in addition 40µl of 0.1M CaCl2 solution was added into both test and control cryovials (called cell-phage mixture) and incubated at 37°C. After 4 hours of incubation, 100µl of cell-phage mixture waspipette out and transferred to luminometer cuvette. Then the D-Luciferin (100 µl) substrate was added to luminometer cuvette and relative light unit (RLU) were measured at 10 seconds integration in luminometer (Model: LB9508 Lumat3; make: Berthold, Germany). Compounds/extracts showing RLU reduction by 50% or more when compared to control will be considered as having antimycobacterial activity. Percentage RLU reduction = Control RLU– Test RLU / Control RLU X 100 (Radhakrishnan et al., 2014). The above mentioned procedure could be applied for drug resistant *M. tuberculosis*.

Anti-Tb Activity Screening By LRP Assay:

Anti TB natural products: Although different types of anti-TB agents are available in world market, there is a growing interest in natural products for novel anti-TB drug discovery, due to non-specific side effects associated with synthetic therapeutics agents and unusual chemical diversity present in natural products. Natural products have been recognized as the source of most active ingredients of medicine. More than 80% of drugs available in world market were derived from natural products or inspired by them. Natural products¬ derived scaffolds are therapeutic templates for the design of new therapeutic drugs using medicinal chemistry and computer-assisted design techniques. Thus, they have a remarkable impact on the treatment of TB in comparison with classical FDA-approved drugs such as rifampicin, kanamycin and cycloserine. Anti-TB compounds isolated from natural sources such as plants, microbes and marine organisms have been found with different skeleton chemical forms and conformations (Conti et al., 2016; Lei et al., 2016).

Medicinal plants: Plants have been used worldwide in traditional medicines for the treatment of various diseases and it is estimated that even today approximately 80% of the world's population rely on medicinal plants as the primary source of medicines (Ekor, 2014). The phytochemical study of some of these plants has yielded a number of active natural products. Next to microorganisms, plants are an important source for anti-TB compounds (Singh et al., 2015). Few studies used LRP assay to screen the plant extracts/compounds for their antimycobacterial properties. Ignacimuthu and Shanmugam (2010) have studied the antimycobacterial activity of two compounds viz.vasicine acetate and 2-acetyl benzylamine obtained from *Adhatoda vasica*.

The results showed that vasicine acetate recorded 99.96%, 97.68 % and 98.93% of RLU reduction against *M. tuberculosis*, drug resistant *M. tuberculosis* and drug sensitive *M. tuberculosis* respectively. Whereas 2- Acetyl benzylamine showed a reduction of RLU by 98.93%, 95.55% and 98.81% in *M. tuberculosis*, drug resistant *M. tuberculosis* and 98.81% drug sensitive *M. tuberculosis*. Antony et al. (2012) have tested the solvent extract of different parts of the plant *Alstonia scholaris* against three different strains of *M.tuberculosis* by using

LRP assay in which the butanol extract of bark showed potential activity against resistant strains at 500µg/ml concentration. Anti-TB activity of ethyl acetate and ethanol extracts of *Sidarhom bifolia* L was tested against *M.tuberculosis* H37Rv and SHRE resistant *M.tuberculosis* at 100µg/ml and 500µg/ml concentration by using LRP assay. Results showed that ethyl acetate extracts have potent antimycobacterial activity whereas ethanolic extract has no activity (Papitha et al., 2013).

Muthuswamy et al., (2013) have tested 32 plants for antimycobacterial activity against *M. tuberculosis* H37Rv, MDR *M. tuberculosis* and sensitive *M. tuberculosis*. Out of 32 plants, 7 plants were shown to have potent activity against three strains of *M. tuberculosis* when tested at 500 µg/ml. Authors concluded that *Ruta graveolens* extract exhibited good antimycobacterial activity against *M. tuberculosis* H37Rv (76.60%), MDR *M. tuberculosis*, (87.25%) and sensitive *M. tuberculosis* (94.32 %) at 100µg/ml concentration. Prabu et al., (2014) have tested the antimycobacterial activity of seven mangrove plants; *Ceriops decandra, Aegiceras corniculatum, Excoecaria agollacha, Avicennia officinalis, Rhizophora mucronata, Suaeda monoica* and *Sesuvium portulacastrum*. They tested both methanol and hexane extracts of all the mangrove plants using LRP assay and the results showed that methanol extracts have good inhibition at 500 µg/ml against the mycobacterial strains tested whereas hexane extract showed less or no inhibitory activity.

The result of the study concluded that the *E. agollacha* possess a significant inhibitory activity among the seven mangrove plants tested against *M. tuberculosis* H37Rv, drug sensitive *M. tuberculosis* and drug resistant *M. tuberculosis*.The various solvent extracts of *Euphorbia hirta* leaves viz. methanol extract, n-Hexane extract and Ethyl acetate extract were screened against *M.tuberculosis* H37Rv at 250 µg/ml and 500µg/ml concentration by LRP assy. The ethyl acetate extract showed 64% RLU reduction at 500µg/ml concentration. In another study, the methanol, ethyl acetate and hexane extract of leaves from *T. procumbens* were tested by LRP assay against *M.tuberculosis* H37Rv at 500 and 250µg/ml concentration. This study revealed that the methanol and hexane extracts have both shown antimycobacterial activity at 500 µg/ml (Rajasekar et al., 2015;2016).

Prabu et al., (2015) have tested the hexane and methanol extract of *Andrographis paniculata* leaves at two different concentrations against *M.tuberculosis* H37Rv, SHRE sensitive *M.tuberculosis* and SHRE resistant *M. tuberculosis*. Among the extracts tested by LRP assay, the methanol extract possess antimycobacterial activity against all the three strains tested. In a study by Suriyamurthy et al., (2016), the antitubercular activity of *Tabubeia rosea* leaves were tested. LRP assay results showed that methanol extract of the leaves inhibited the standard and clinical isolate of *M.tuberculosis* at 500µg/ml concentration. Muthuselvi et al., (2017) have screened the ethyl acetate extract obtained from the bark of *Cassia marginata* against *M.tuberculosis* H37Rv and SHRE resistant *M.tuberculosis* at 100 µg/ml and 500µg/ml concentration. The extracts demonstrated inhibitory activity at whereas against the SHRE resistant strain the extract showed inhibitory activity at 100 µg/ml and against all strains tested at 500 µg/ml. Plumbagin is an organic compound obtained from *Plumbago zeylanica*. Three compounds have been derived from Plumbagin and tested for antimycobacterial activity against two clinical isolates of drug sensitive *M. tuberculosis* and *M. tuberculosis* H37Rv. Of these three compounds, two showed activity at 50 and 100 µg/ml (Nayak et al., 2014).

MI et al., (2018) investigated the methanolic extract of *Dendropthoe falcata* leaves for anti TB activity against *M.tuberculosis* H37Rv, all sensitive MTB and MDR-MTB by LRP assay was done at concentrations 100 and 500 µg/ml concentration. Test drug showed inhibition of H37Rv and all sensitive *M.tuberculosis* and no inhibition with MDR *M. tuberculosis*. The Minimal Inhibitory Concentration (MIC) of *Nigella sativa* seeds were determined against all three mycobacterial strains using Luciferase Reporter Phage (LRP) assay. In this study, the methanolic and water extract of *N. sativa* seeds showed inhibition against *M. tuberculosis* H37Rv, all drug sensitive *M. tuberculosis*, MDR*M. tuberculosis* respectively. The methanol extract showed least inhibition at concentration of 50 µg/ml, 250 µg/ml and 100 µg/ml against *M. tuberculosis* H37Rv, all drug sensitive *M. tuberculosis* and MDR *M. tuberculosis* respectively (Anbarasu et al., 2018).

Quercetin and rutin, two flavonoids were examined for antimycobacterial activities against *M. tuberculosis* H37Rv (ATCC 27294). The quercetin exhibited (99.30 ± 0.268%) in (LRP) assay at 200 µg/ml and 56.21 ± 0.97% inhibition in (BMD) at 50 µg/ml, whereas rutin exhibited (90.40 ± 0.68%) in LRP assay at 200 µg/ml and 56.10 ± 0.67% inhibition in BMD at 50 µg/ml. The minimum inhibitory concentration (MIC) was found to be 6.25 µg ml−1 and 25 µg ml−1 respectively. The current investigation suggests that quercetin has better inhibitory activity than rutin (Sasikumar et al., 2018). The methanol extract of *Solanum torvum* and ethyl acetate extract of *Vitex negundo* and *Zingiber mauritiana* were also screened for anti-tubercular activity against *M. tuberculosis* H37Rv using Luciferase Reporter Phage (LRP) assay. All the three extracts were exhibited anti TB activity at 500 µg/ml concentration. In particular, the *S. torvum* extract showed 98.46% inhibition (Vaishnavi et al., 2020).

Marine organisms: The potential of marine organisms is well documented in the recent past. Yet, their utility for anti-TB drug discovery is still in its infancy. Until 2000, there are only two reports of in-vitro anti-TB activity from marine origin. There are very few anti-TB compounds isolated from marine macro organisms such as molluscs (kahalalides A and F), sponges (heteronemin), corals (litosterol) (De Souza et al., 2006). Bioactive substances from natural sources are available in extremely low quantities leading to limitations in using the reservoir of marine organisms for bioassay and therapy. To overcome these problems, few methodologies such as mariculture,

bioreactors, sponge cell culture, genetic modification and most importantly chemical and semi-synthetic approach can be pursued (Lindequist, 2016). Certain anti-TB compounds produced by marine sponges (agelasine) and corals (litosterol) have been synthesized by chemical methods (Mancini et al., 2007). Unfortunately, none of the several hundreds of non-microbial natural products with antimycobacterial activity have moved forward in drug development.

Amudha et al.,(2015) tested hexane and ethanol extracts of *T. conoides* against *M. tuberculosis* at 100µg/ml and 500µg/ml concentration by LRP assay. Both solvent extracts showed significant inhibitory activity at 500µg/ml concentration. Mayakrishnan et al. (2017) have screened the red algae, *Kappaphycus alvarezii* for antimycobacterial activity. They tested the extracts of acetone, chloroform and ethanol against *M. tuberculosis* H37Rv and clinical isolate of *Mycobacterium tuberculosis* by LRP assay. The acetone and chloroform extracts showed antimycobacterial activity against *M. tuberculosis* H37Rv at 500µg/ml concentration whereas all the three extracts showed antimycobacterial activity against clinical isolate of *M. tuberculosis*. Sundar et al., (2018) reported the antimycobacterial activity of *Sargassum swartzii* against the whole cell *M.tuberculosis* H37Rv by LRP assay. Among the extracts tested, the methanol extract, ethyl acetate, chloroform extract and aqueous extract showed inhibition at 500 µg/mL concentration against *M. tuberculosis* H37Rv whereas, n-hexane showed no inhibition against the strain tested.

Microorganisms: Microorganisms that live together in the environment develop long-lasting methods to keep each other at bay. As a result, many of our most effective bactericidal agents have come from environmental organisms. Microbes are the most exploited sources for bioactive natural products including anti-TB compounds. To date, more than 1000 antimycobacterial compounds have been reported from microbial sources among which actinomycetes are the best reported microbial source. More compounds from actinobacteria of terrestrial and marine origin are still in different stages of investigation to be developed as potential anti-TB drugs. Some of the reports on anti TB activity of actinobacteria by LRP assay are described below. Radhakrishnan et al., (2010) screened bioactive extracts from 15 actinobacterial strains isolated from rare marine and forest ecosystems by LRP assay, for the first time.

Culture supernatant and mycelia were extracted with ethyl acetate and methanol, respectively. Culture filtrates and crude extracts were tested against standard strain *Mycobacterium tuberculosis* H37Rv and drug sensitive and drug resistant clinical isolates of *M. tuberculosis* by luciferase reporter phage (LRP) assay. Considerable variation was observed in antimycobacterial activity between actinobacterial culture filtrates and solvent extracts. Actinobacterial strains viz., D10, D5 (desert), CSA14 (forest), CA33 (alkaline soil), NEK5 (Neem plant), MSU, ANS2, R2 and M104 (marine) screened in the present study were found to be highly potent showing good antibacterial and antimycobacterial activity. Five of them; A3, CSA1, EE9, ANS5 and R9 were exclusively active against *M. tuberculosis*. Secretary products of actinobacteria of rare ecosystems are meant to antagonize organisms in their respective environments. These are likely to be novel antimycobacterial compounds as they unknown to human pathogens.

Bioactive potential of actinobacteria isolated from certain less explored Indian ecosystems was tested against *Mycobacterium tuberculosis* and other non mycobacterial pathogens. Actinobacteria were isolated from the soil samples collected from desert, coffee plantation, rubber forest, and hill area from Western Ghats and Eastern Ghats Ecosystems in India and their cultural and micromorphological characteristics were studied. Crude extracts were prepared by agar surface fermentation and tested against *M. tuberculosis* isolates by luciferase reporter phage (LRP) assay at 100 µg/mL. Activity against nonmycobacterial pathogens was studied by agar plug method. A total of 54 purified cultures of actinobacteria including 43 *Streptomyces* and 11 non *Streptomyces* were isolated. While screening for antitubercular activity, extracts of 39 actinobacteria showed activity against one or more *M. tuberculosis* isolates whereas 27 isolates exhibited antagonistic activity against nonmycobacterial pathogens. In particular crude extracts from sixteen actinobacterial isolates inhibited all the three *M. tuberculosis* isolates tested. Findings of the present study concluded that less explored ecosystems investigated in this study are the potential resource for bioactive actinobacteria (Radhakrishnan et al., 2011; Manikkam et al., 2014).

Extracellular pigment extracted from the forest soil *Streptomyces* sp SFA5 using ethyl acetate was tested for antitubercular activity against *M. tuberculosis* H37Rv by LRP assay. The crude pigment showed activity against *M. tuberculosis* H37Rv at 250 µg/mL concentration (Manikkam et al., 2016). Gopikrishnan et al., (2017) studied the MIC of quercetin molecule purified from *Streptomyces fradiae* PE7 at 100 to <1 µg/ml against *M. tuberculosis* H37Rv, clinical drug sensitive *M. tuberculosis* and multi drug resistant (MDR) *M. tuberculosis* strains by adopting LRP assay. Quercetin showed more than 65% reduction against all the three *M. tuberculosis* strains at 3.1 µg/mL concentrations.

The crude extracts from 15 marine actinobacterial strains isolated from Andaman and Nicobar Islands were tested against the standard strain *M. tuberculosis* H37Rv, clinical drug sensitive *M. tuberculosis*, and MDR *M. tuberculosis* strains by luciferase reporter phage (LRP) assay at 500 µg/ml concentration. Among the 15 extracts that were tested for anti-tubercular activity, the crude ethyl acetate extract of the 14 actinobacterial strains showed anti-tubercular activity against at least one of the three *M. tuberculosis* strains. Exceptionally, the ethyl acetate extract of strain SACC 168 inhibited all three *M. tuberculosis* strains tested (Manigundan et al., 2019). A study by Ameer et al., (2020) have extracted antitubercular protein from *Staphylococcus*

hominis which significantly inhibited the growth of *M. tuberculosis* with a RLU reduction of more than 90% against *M. tuberculosis* H37Rv.

Synthetic Compounds: Sivakumar et al., (2010) have synthesized 25 chalcone derivatives based on the Claisen-Schimdt scheme and tested their antimycobacterial activity at two different concentrations. Among them, a compound, designated as C24, has shown 99% reduction against *M. tuberculosis* H37Rv. In a study, seven 2r,4c-diaryl-3-azabicyclo[3.3.1]nonan-9-one N-isonicotinoylhydrazone derivatives have tested their antimycobacterial activity against *M. tuberculosis*. All seven derivatives were found to possess antimycobacterial activity with the range of 62-85% of inhibition at both 1 and 2µg/ml concentration (Sankar et al., 2010). Twelve derivatives of 2-methyl-1H-benzimidazole hydrazide have screened against *M. tuberculosis* H37Rv.

Out of 12 derivatives, 7 derivatives showed significant reduction in RLU at both 50 and 100µg/ml concentration (Uma et al., 2009). In another study, Kanagarajan et al., (2011) have synthesized 6 novel 1,1'-(5,5'-(1,4-phenylene) bis(3-aryl-1H-pyrazole- 5,1-(4H,5H)-diyl))diethanones from bis chalcones and assessed their antimycobacterial activity against *M. tuberculosis* H37Rv and INH resistant *M. tuberculosis*. They found all the six compounds possess inhibitory activity against both the strains at 1 and 2µg/ml concentration. Kumar et al., (2011) have synthesized a series of quinolone coupled 1,2,3-triazoles compounds and tested their antimycobacterial activity by LRP assay. They reported that one compound has shown Inhibitory activity against *M. tuberculosis* at 5 and 25µg/ml concentration.

Durgad et al., (2012) have synthesized 9 benzimidazole derivatives with the intermediate chalcones and results found that 4 compounds possess potent antimycobacterial activity against *M. tuberculosis* H37Rv strain. Mohan et al., (2012) synthesized the various derivatives of 1,2,3,4-tetrahydropyrimidine-5-carbonitrile based on the bioisosteric similarities of Isoniazid. They selected compounds based on molecular docking and were tested for their antimycobacterial activity against *M. tuberculosis* H37Rv and drug resistant *M. tuberculosis* using LRP assay. Among the five test derivatives, three of them showed potential antimycobacterial activity at 500µg/ml concentration. Derivatives of 2-(4-methylpiperazin-1-yl)-N-(4,6-diarylpyrimidin-2-yl)acetamides have tested their antimycobacterial activity against *M. tuberculosis* H37Rv and INH resistant *M. tuberculosis*. Results of the study found all 9 derivatives showed inhibitory activity against the strains tested at 1 and 2µg/ml concentration (Kanagarajan and Gopalakrishnan, 2012).

Similarly, Narender et al. (2016) have synthesized 22 derivatives of 3-substituted-7-benzyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one and 3-substituted-7-benzyl-2-methyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one and screened their antibacterial activity. Based on their inhibitory effect, five of them were tested for their antimycobacterial activity against a standard and clinical isolate of *M.tuberculosis* strains. Among them, two of the derivatives have showed potential antimycobacterial activity at less concentration. Two hydrazones, Benzoic acid (4-allyloxybenzylidene)-hydrazide and 4-chloro-benzoic acid (4-allyloxybenzylidene)-hydrazide have been synthesized and tested against *M.tuberculosis* H37Rv in which the latter found to possess significant antimycobacterial activity at both 100 and 200µg/ml concentration (Therese and Geethamalika, 2017). Brindha et al. (2017) have demonstrated the repurposing of drug for tuberculosis treatment. They screened 1554 known drugs by docking studies. Based on their potential in docking studies, they have selected five potential drugs and screened them by LRP assay. They found that two drugs lymecycline and cefpodoxime has potential inhibition at 20µg/ml concentration against drug sensitive and drug resistant *M. tuberculosis* strains.

Nanoparticles: In vitro antitubercular activity of isoniazid (INZ) loaded solid lipid nanoparticles (SLNs) and free isoniazid (INZ) was evaluated by LRP assay against *M. tuberculosis* H37Rv and MDR MTB. Results of LRP assay in H37Rv strain showed that percentage reduction in relative light unit (RLU) for INZ-SLNs and free INZ were 99.75 and 99.898% respectively, whereas in case of INZ resistant strain they were found to be 90.27 and 90.52% respectively, confirming notable antitubercular activity (Mohanta et al., 2018).There are few studied which have reported the anti TB activity of nanoparticles and drug loaded nanoparticles by adopting LRP assays. Green synthesis of silver chloride nanoparticles (AgCl NPs) using commercial yeast extract has been carried out. The physico–chemical characterizations of AgCl NPs were carried out by UV–visible spectroscopy, XRD, FTIR, HR-SEM equipped with EDAX and TEM.

In vitro efficacy of anti-mycobacterial properties of AgCl NPs were determined by agar well diffusion and Luciferase Reporter Phage (LRP) assay were used against *M.smegmatis* (MC^2155) and *M.tuberculosis* H37Rv respectively. This shows that the AgCl NPs have potential anti-myobacterial activity against *M. tuberculosis* H37Rv (Sivaraj et al., 2020). A recent study by Govindaraju et al. (2020) synthesized nanoparticles using seaweed *Turbinaria ornata* and found that it has the ability to inhibit the growth of *M. tuberculosis* strain by 73% of RLU reduction. The characterization of these nanoparticles have also been studied with spectral and microscopic analysis.

CONCLUSION

The review of published literature on anti TB screening assays revealed that more actinobacterial extracts were screened for anti TB activity than other sources by adopting LRP assay. Furthermore, the mycobacteriophage constructs used in all the studies had narrow host range and mainly infect *M. tuberculosis*, limiting utility. Developing new mycobacteriophage constructs with wide host range may widen the utility of LRP assay for drug discovery against non tubercular mycobacterial

pathogens.LRP may help to reduce the time required for anti-TB screening compounds against various sub-population of *M. tuberculosis* and helps to screen large number of compounds as it involves less quantity of compounds as well the turnaround time to detection reduced from weeks to days which enables to accelerate the screening for new antituberculosis natural/synthetic compounds.

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IoT Based Pollution Monitoring System for Effective Industrial Pollution Monitoring and Control

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ABSTRACT

This platform relies on an IoT and a cloud computing technology to watch Industrial Pollution Monitoring and control the quality of environment in anywhere and anytime. An IoT-based system to monitor and control the pollutants andthereby helping them to moderate their trial on exposure topollutants. Industrial pollution monitoring is the collection of data at different locations of industries and at regular interval frames so as to give the information which might be utilized to characterize current conditions. Pollution Monitoring and controlling System demonstrates an efficient utilization of technology by which screen and report environmental parameters like gas, smoke and temperature and humidity. An efficient environmental monitoring system is required to monitor and assess the conditions just in case of exceeding the prescribed level of parameters (e.g., noise, CO and radiation levels). A solution for monitoring the noise and air pollution levels in industrial environment or particular area of interest using embedded computing system is proposed. This paper proposes an approach to build a cost effective standardize pollution monitoring device using the wireless technology (i.e.) Internet of Things (IoT) and a cloud computing technology.This work discusses the implementation of cloud based IoT system for air quality monitoring which is accessible as a web interface as well as in a type of an android application.

KEY WORDS: AIR POLLUTION MONITORING, CLOUD COMPUTING, ENVIRONMENTAL POLLUTION, HEALTH CARE IOT ANALYTICS.

INTRODUCTION

Atmospheric conditions keep on weakening every year because of development of civilization and increasing unclean emissions from industries and automobiles. Despite the fact that air is a vital asset forever; numerous individuals are unconcerned with the seriousness of air contamination or have as of late perceived the issue. Air pollution has surprised the world and is being unmistakably examined on the grounds that it has adjusted the environmental cycle like anything. Although air is an indispensable resource for all times, many of us are indifferent to the severity of pollution or have only recently recognized the problem (M.de Boer., 1998, Van Egmond., 1998, Sengupta et al., 2019). Among various types of pollutions like water, soil, thermal, and noise, air pollution is the most dangerous and severe, causing climate change and life-threatening diseases. Air pollution is one among the main players, leading to global climate change which has caused anomalies within the temperature pattern, crops production and

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has led to advent of newer diseases causing widespread devastation.

The wellbeing impacts of contamination are exceptionally extreme that cause stroke, lung malignant growth, what's more, coronary illness. Moreover, air poisons have a negative sway on people and the world's biological system, as watched in late worldwide air contamination issues like ozone exhaustion (Kulkarni and Zambare, 2018, Rout et al 2018, Saha et al., 2017). According to the World Health Organization (WHO), 90 percent of the populace presently inhales contaminated air, and air contamination is the reason for passing for 7 million individuals consistently (World Health Organization., 2011, Dhingra et al., 2019).

As tremendous research is going on in these serious issues to monitor and control the quality of air, the main purpose of this system is to estimate the quality of air for people and any other living thing which exists on the earth. The proposed embedded system model includes various sensors like MQ135 (sensitive to CO2, NOx, SO2, NH3, and benzene), Temperature and humidity sensor and noise level detector. It takes information about the surrounding environmental parameters such as Gas, Smoke and temperature and humidity through sensors and uploads it directly to the internet and determine the quality of environment so that preventive measures can be taken.

MATERIAL AND METHODS

In recent years, introduction of technologies just like the Internet of Things (IoT) and cloud computing has revealed new capabilities of real-time monitoring in various fields. Thus, many scholars have studied integrating these technologies to indoor air quality monitoring system (Liu et al., 2011). With air contamination getting rudimentary and arrangement of mechanical advances throughout the years have sanctioned people to establish their own checking framework that too at moderate cost and have had the option to surmise a characterizing job in the manner by which air contamination is being checked. Amalgamation of advanced mobile phones with IoT has additionally solidified the job of IoT and has taken unmeasurable steps in the field of Bio clinical applications (Sengupta et al., 2019).

However,these studies were only focused on integrating anarchitecture of IoT platform to monitor the air quality in Realtime. Since the technologies feature a wireless sensor network to automatically transmit, process, analyze, and visualize data, merging these new technologies also can also offer great advantages to enhance indoor air quality. Therefore, an IoT-based indoor air quality monitoring platform based on integration of cloud computing andIoT is presented in this research.An experimental wireless system that extends beyond the hotspots capabilities to supply wireless connectivity at distant areas and at a coffee cost. The system combines the paradigm of Wireless Mesh Networks with the Captive Portal technology to provide an outsized varies Internet-based communication services and applications. Finally, some experiments in terms of context services and traffic modelling, and demonstrate that the developed system can be easily deployed in terms of coverage, management, and offered services, (Sengupta et al., 2019, Marques et al 2019).

The pollution Dynamic Monitoring System has also been worked upon previously and there is a need of development of a smart environment monitoring system with IoT support to know the related information on the mobile itself. The rest of the paper is organized as follows; Section 3 discusses the existing systems and the need to present the proposed system followed by section 4 which presents the overview of an air pollution monitoring system. Section 5 speaks about the software implementation of IOT and section 6 presents the hardware description. Result and its discussion are presented in section 7 followed by conclusion.

Existing System: Existing devices used for pollution monitoring needed manual collection and processing of knowledge continuously from time to time which successively needs a group staff to continuously monitor and log the info. A model which has been created and it screens the changeability of boundary like Air, Noise, Temperature, Humidity and lightweight. Existing devices used for pollution monitoring needed manual monitoring of the device over continuously from time to time which successively needs a group of staff to continuously monitor the data and log into the information (Tastanand and Gokozan., 2019).

Manual systems put pressure on people to be correct altogether details of their work on all times, the matter being that people aren't perfect. With manual systems the extend of service depends on individuals and this puts a requirement on management to run training continuously for the workers to keep them motivated and to make sure they are following the right procedures. It is oftentoo easy to accidentally switch details and find yourself with inconsistency in data entry or in hand written orders. Also, person responsible of knowledge logging may change the info as instructed by higher official's makes it unreliable.

Existing System Disadvantages: Need of manual labor, cost of operation is high, has the risk of human error and data can easily altered.

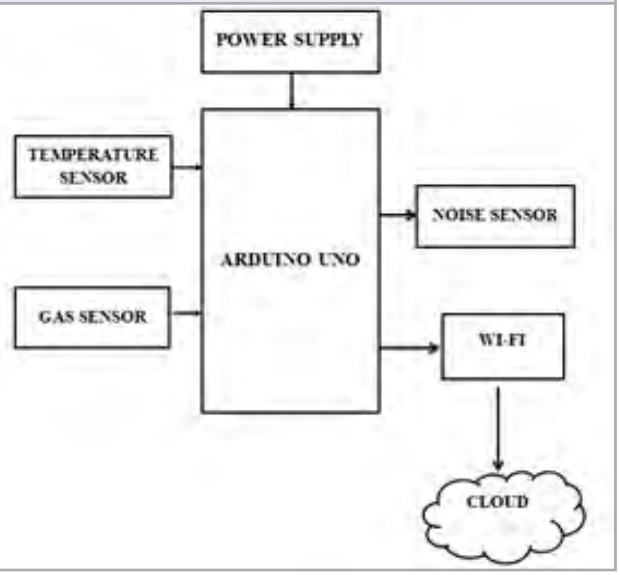
Proposed System: The proposed work, uses locally available gas sensor for observing the polluted gases like Carbon monoxide (CO), Carbon dioxide (CO_2) and parameters like temperature, humidity and sound. The sensors data can be uploaded to the cloud and if any of these exceeds a value then an email will be sent to the pollution board authority.The pollution authority can view the data from a remote location using an android app and he can also shut down the entire industry from a faraway location if need arises.The access to the data is secured with the help of encryption systems.A mobile application originally developed to make the proposed

IoT system with features of anytime, anywhere. The device has been tested for reliability of the data and the platform has been implemented in a building to test its feasibility. The figure 1 shows the block diagram of this proposed system.

Hardwares Used: ArduinoUNO, Temperature and humidity sensor,Gas sensor, Noise sensor, Power supply, Wi-Fi, Cloud Softwares Used :Arduino IDE, Hyperterminal

Block Diagram:

Figure 1: Block diagram



Software Implementation For IoT

Cloud Computing Service: Cloud computing service involves development of functionality to manage the sensors where it might be required to coordinate between the Sensor Owner, the cloud provider and also the user. The sensor owners develop the system and develop functionality to publish the information in the cloud. The cloud providers then provide the cloud services and will charge the top user with some fee for the access. So as to avoid PaaS is used (Platform as a Service). PaaS provides a platform for software creation and is delivered over the net, and provides developers the liberty to think about building the software while still not having to worry about operating systems, software updates, storage, or infrastructure.

Android Service: Android has changed into a highly popular software operating system operating in voluminous of smartphones. Android scores in terms of user-friendly features and services. Many companies are using android software system for his or her smartphone device to produce high end features to their users. The Proposed system aims in designing a well-built system that monitors real time emission levels and temperature of all the Industries and required areas, store all the collected data in and analyze them in cloud using Internet of Things. This method uses various sensors like temperature and humidity sensor,MQ-135 sensor, noise sensor to measure various parameters like temperature, humidity, gas, noise respectively.

Arduino microcontroller is used which uses Reduced Instruction Set (RISC) program that reduces the complexity of the code significantly. WIFI module is utilized to store the data within the cloud which is flexible and simple to attach and it's connected through the hotspot. The information are often viewed in any browser including smart phones by logging in using the credentials.For Air Quality Index to be calculated there's a requirement to record the concentration of minimum three pollutants out of which one amongst should be PM10 or PM2.5.Thus, for the proposed system include Sulphur Dioxide (SO2), Nitrogen Dioxide (NO2), Particulate Matter (Size less than 10μm) or PM10 such that we are ready to calculate the AQI correctly and that we include temperature sensor to live the environmental parameters too. The project uses the concept of IoT for monitoring and controlling the system employing a ThingSpeak cloud server.

The Node MCU is configured to transfer the measured data of all sensors on a distant server. The net application provides global access to the measured data using any device that has internet connection capability. Data collected from the sensors are analyzed and passed the data within the sort of a string to update the net page within the remote server.The system also uses an android app called Industrial Pollution Monitoring System which shows the sensors data graphically. The proposed system employs Arduino together with Sulphur Dioxide (SO2), NO2 and PM-10 Sensor along with the temperature and humidity sensor to attain the task of air quality monitoring. The sensor senses the worth and with the assistance of Arduino forwards the value to the cloud. The info management module at the cloud additionally at the App suitably disseminates the knowledge to the specified users.

Hardware Discription: Arduino UNO:This module are often used for security, switch, and monitoring applications. Its accuracy is often easily adjusted for the convenience of usage. It uses a microphone which supplies the Arduino Uno.

Figure 2: Arduino UNO

In Figure. 2 Arduino UNO is exposed to source podium which involves of both a physical programming circuit board (Micro controller) and a bit of software (Integrated development Environment)

Temperature and Humidity sensor [DHT11]:

Figure 3: Temperature and Humidity sensor

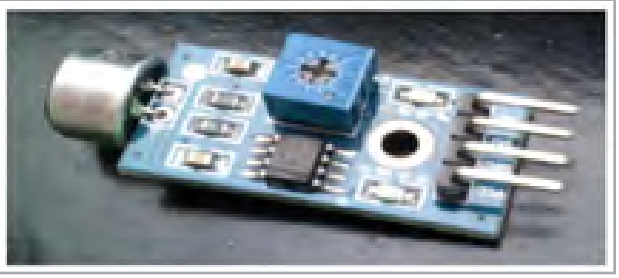
DHT11, a Humidity and Temperature Sensor generate calibrated digital output. DHT11 are often interfacing with any microcontroller like Arduino, Raspberry Pi, etc. and get instantaneous results. DHT11 may be a low-cost humidity and temperature sensor which provides high reliability and long-term stability. In Figure 3we infer that the sensor consist of 4 pins namely Vcc, Data pin, Nc, and GND respectively.

Gas Sensor

Figure 4: Gas Sensor

In current technology scenario, monitoring of gases produced is extremely important. From home appliances like air conditioners to electric chimneys and safety systems at industries monitoring of gases is extremely crucial. Thus gas sensors play a major role in industrial pollution monitoring. Figure 4 is a basic gas sensor used in most of the gas detecting equipments. Sound Sensor Module: The sound sensor module provides a simple thanksto detect sound and is usually used for detecting sound intensity. The sound sensor module consists of a microphone, an amplifier, a peak detector and a buffer. Figure.5 is a sound sensor module that detects a sound, processes it as an output voltage and finally sends to the microcontroller which performs the necessary process.

Figure 5: Sound Sensor module



Internet of things: The Internet of Things (IOT) is revolutionizing and improving the way of work and live but it's only possible with pervasive, flexible and long-lived wireless property. At the center of it all may be a small device known as the IOT Module that's liable for connecting nearly something to wireless networks. IOT Modules associate with a large vary of wireless technology standards and that they give a spread of options which will impact the success of IOT applications.

Power supply: The AC voltage, usually 220V, is linked to an electrical device that stages that AC voltage down to the total of the measured DC output. A Diode rectifier then provides a full wave improved voltage that's at the start filtered by a modest capacitor filter to supply a DC voltage. This successive DC voltage mostly has particular ripple or AC voltage difference. A controller circuit removes the wave and moreover remains equal DC value despite the fact that the input DC voltage varies, or the load connected to the output DC voltage changes. This voltage regulation is typically obtained exploitation one of the favored transformer IC units.

RESULTS AND DISCUSSION

The aim of this technique is to assist in reducing respiratory problems related to industrial activities and to watch the level of pollution. The main focus is on finding solutions to the increasing problem of harmful gasses amounting from industrial practices in the country. In this work, sensors were implemented and used to detect the presence of undesired gases in the air of residential areas near factories and industrial activities.Figure.6 shows the various parameter (gas, humidity, temperature and noise) values that are collected and displayed.

Figure 6: LCD display

The sensors provide continuous monitoring and record data for air pollution continuously, where these results are reported in the figure 7- 10 and analyzed using Internet of Things technology.

Figure 7: Login page

Figure 8: Parameters Values

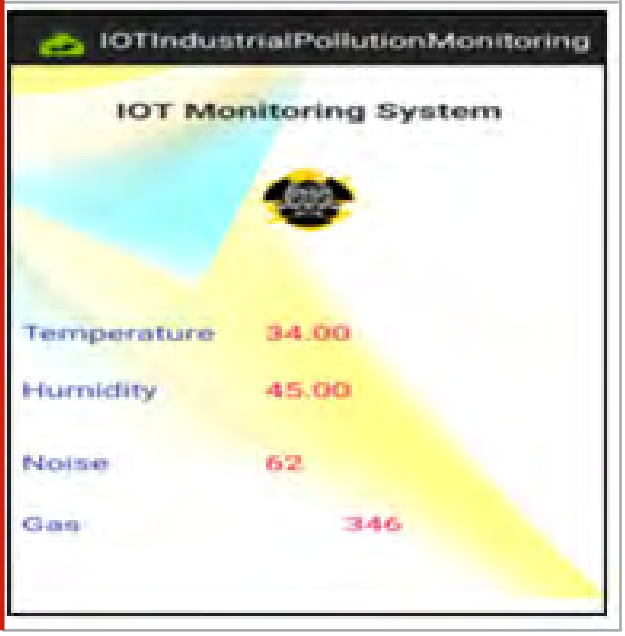
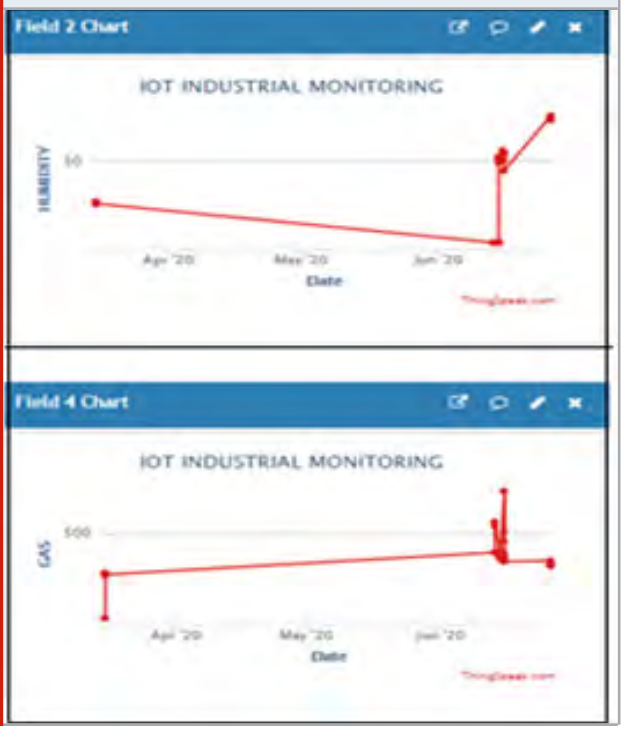


Figure 7 frontend of the software and Figure 8. shows the obtained results. Figure 9 and 10 shows the parameters variation in the month of April, May and June 2020. From this figure, it has been observed that the proposed method yields good results and the data's which are stored in the cloud can be retrieved when and wherever required. Also using IoT technology, the parameters can be viewed by mobile phone.

Figure 9: Temperature and noise flow

Figure 10: Humdity and Gas flow chart



CONCLUSION

The system to watch the air of environment using Arduino microcontroller, IOT Technology is proposed to measure quality of air. With the utilization of IOT technology, the method of monitoring various aspects of environment like air quality monitoring issue can be done and it has been retrieved when and wherever it is required. The MQ135 gas sensor gives the sense of various sorts of dangerous gases and Arduino is the heart of this mechanism which controls the whole process. Wi-Fi module connects the entire process to internet and LCD is employed for the visual Output. The Automatic Air & Sound management system may be a breakthrough to contribute an answer to the most important threat. The air and sound monitoring system overcomes the matter of the highly-polluted areas which may be a major issue. Moreover, it is an auto monitoring system; the major advantage of this is to reduce the involvement of human being in the hazardous environment and assures safety also. It supports the new technology and effectively supports the healthy life concept. This system has features for the people to watch the quantity of pollution on their mobile phones using the appliance.

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Functional Features of Hemostasis in Weakened Newborn Calves Treated with Aminosol

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ABSTRACT

With many negative changes in the body, the development of hemostatic system activity disorders with the formation of thrombophilia is possible. The high frequency of occurrence of physical weakening of newborn calves creates a risk for them to activate the hemostatic system, which requires a search for approaches to eliminate the asthenic state and optimize the activity of hemostasis. The study examined 34 weakened newborn calves that were obtained from first-born cows. To correct their condition, the calves were given aminosol in a generally accepted dose. Initially, animals had an increase in antioxidant protection of blood plasma with a decrease in the severity of lipid peroxidation processes in it. Weakened calves showed a high activity of the coagulation system of blood and platelets and decreased hemostatic capabilities of the vascular wall. As a result of the use of aminosol in weakened newborn calves, there was a significant increase in the activity of their antioxidant system, leading to the containment of lipid peroxidation processes in their blood, reduced functional readiness of platelet and plasma hemostasis and increased hemostatic capabilities of their vessels. Perhaps the continued use of this drug can lead to the normalization of the considered indicators at a longer observation time..

KEY WORDS: HEMOSTASIS, NEWBORN CALVES, AMINOSOL, PLATELETS, BLOOD VESSELS..

INTRODUCTION

Modern conditions of animal husbandry increasingly dictate the need for its intensification (Vorobyeva and Medvedev, 2020a; Glagoleva and Medvedev, 2020). However, great attention must be paid to the productive capabilities of first-calf heifers. Not in all cases, first-calf cows are capable of producing highly viable offspring. This is largely due to their insufficient body weight and sometimes early insemination (Oshurkova and Medvedev, 2018a). For this reason, calves obtained from them often have hypotrophy, negatively acting in them on metabolism, and therefore on growth in their body weight (Glagoleva and Medvedev, 2018).

It is known that with so many negative changes in the state of the animal's body, a disturbance in the activity of the components of the hemostatic system, causing the formation of thrombophilia, is possible (Vorobyeva and Medvedev, 2018; Mal et al., 2018a). A sufficiently high frequency of occurrence of weakening of the physical condition in newborn calves provides them with a risk of a high frequency of occurrence of episodes of activation of components of the hemostasis system (Vorobyeva and Medvedev, 2019; Vorobyeva and Medvedev, 2020b) with poor study of the potential of available approaches to eliminate the asthenic state with regard to the effect on hemostasis activity.

Of great scientific and practical interest is the assessment of the effect on weakened newborn calves of various

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means of increasing their viability, taking into account the severity of the effect on hemostasiopathy taking place against the background of physical weakening. Of particular interest is the assessment of the effect of the aminosol preparation, which is often used in newborn calves born to weakened, on the parameters of the hemostatic system. In the present study, the goal was set to assess the nature of the effect of the aminosol agent on the hemostatic system in weakened newborn calves.

MATERIAL AND METHODS

This work was carried out in accordance with the ethics defined by the European Convention for the Protection of Vertebrates, which is used for scientific purposes (adopted at Strasbourg on 18 March 1986 and confirmed at Strasbourg on 15 June 2006). Material for the article was obtained during a survey of 34 newborn calves born weakened, but with normal body weight. The control group consisted of 25 healthy newborn calves. The animals were examined to determine the level of lipid peroxidation in plasma, which was estimated by the amount of thiobarbituric acid-active products in their plasma using the Agat-Med kit (Russia) and the level of acylhydroperoxides when registering the plasma antioxidant potential blood (Volchegorsky et al., 2000). The platelet content in the blood of animals was determined using a Goryaev's camera. The expressiveness of the aggregation of platelets (AP) was defined, applying a visual micromethod of its registration (Shitikova, 1999) with a number of inductors: ADF (0.5×10-4 M), collagen (cultivation 1:2 main suspensions), thrombin (0.125 pieces/ml), ristomitsiny (0.8 mg/ml), adrenaline (5×10-6 M) in plasma rich with platelets after it was standardized on the maintenance of platelets to concentration 200×109 of platelets in one liter.

The ability of vessels to inhibit platelet aggregation was recorded by applying a time venous occlusion sample (Baluda, 1987) based on an AP evaluation in visual micrometode (Shitikova, 1999) to all inductors used. The value of indices of anti-aggregation activity of vascular wall with respect to all inductors used in operation was determined. For this purpose, the value of the time of development of AP in plasma, which was obtained under conditions of temporary venous stagnation for the time of development of AP in plasma obtained outside it, was divided. The index of anticoagulation activity of the vessel wall was calculated by dividing the activity of antithrombin III in plasma (Barkagan, 1999) taken using a venous occlusion sample by the value of its activity before it in plasma obtained without applying a cuff to the vessel (Baluda, 1987). Vascular control of fibrinolysis was determined by calculating the value of the fibrinolytic activity index of the vascular wall. This was done by dividing euglobulin lysis index (Barkagan, 1999) in intact plasma by its index in plasma taken after temporary vascular occlusion (Baluda, 1987).

Coagulation hemostasis was evaluated by the values of activated partial thromboplastin time, prothrombin time and thrombin time (Barkagan, 1999). Correction of functional indices in weakened newborn calves was carried out with aminosole agent (manufactured by "Biofactors," Czech Republic) at a dose of 8 ml/day, used in the form of evaporation per head for 8 days. Determination of all taken into account parameters in control animals was carried out once, in weakened animals - twice - at the moment of taking into study and on the next day after completion of correction. Statistical processing of the obtained data in the work carried out was carried out using the Student 's t-criterion.

RESULTS

In the outcome, weakened calves showed signs of weakness, sluggishness under reduced interest in all elements of reality. In the plasma, the weakened animals had a higher content of acyl hydroperoxide (3.42±0.19 D233/1 ml) and products capable of reacting with thiobarbituric acid (5.02±0.16 μmol/l) under depression of the antioxidant possibility of their plasma (23.2±0.34%). These parameters in the control calves were 1.42±0.07 D233/1 ml, 3.48±0.10 μmol/l and 34.2±0.26%, respectively. The number of platelets in the blood of the weakened calves was within the limits of the generally accepted norm (table 1). The development time of AP in these calves was significantly reduced. Previously, AP occurred in them under the action of a collagen inductor (accelerated with respect to the control level by 69.1%), a little later when using an ADP inductor (accelerated with respect to the control level by 64.3%) and a ristomycin inductor (accelerated with respect to the control level by 44.3%). AP in response to thrombin developed even later (accelerated relative to control level by 45.9%). The most delayed in weakened calves AP developed under the influence of adrenaline (accelerated compared to control by 44.9%).

In weakened young animals, a decrease in the levels of the indices of antiaggregation activity of the vascular wall was found for all applied inducers. The index of anti-aggregation activity of the vascular wall with collagen turned out to be the lowest, the index of anti-aggregation activity of the vascular wall with adrenaline and thrombin was slightly higher, and the index of anti-aggregation activity of the vascular wall against ADP and ristomycin was even higher.

The vessels of weakened newborn calves showed a decrease in the ability to control coagulation by 19.6%, which was assessed by a decrease in their index of anticoagulation activity of the vessel wall. The weakening of their fibrinolytic activity of blood vessels amounted to 18.6%, judging by the magnitude of the index of fibrinolytic activity of the vascular wall. Physically weakened calves had high values of blood coagulation developing in external (37.5%), internal (43.0%) and final stage fibrin formation (20.3%) earlier than in the control group. Aminosol evaporation was accompanied in weakened calves by activation of their general condition and improvement of hematological indices recorded in operation. In aminosol-treated

calves, the plasma concentration of acyl hydroperoxides (to 1.65±0.25 D233/1 ml) and compounds capable of reacting with thiobarbituric acid (to 3.81±0.39 μmol/l) decreased by increasing the antioxidant properties of their plasma (to 29.8±0.07%).

The correction to the weakened calves provided them with a pronounced slowdown in the AP. This was manifested by the later platelet response of these calves to the addition to platelet-rich plasma of all the aggregation inductors tested in operation. In weakened calves, as a result of the correction, an increase in the values of the indices of antiaggregatory activity of the vascular wall in response to all applied inductors occurred. The smallest was the value of the index of antiplatelet activity of the vascular wall in the case of collagen. Other indices of antiplatelet activity of the vascular wall were higher, also having a tendency to approach the control. Weakened calves given aminosol showed an increase in vascular control over plasma hemostasis, judging by an increase in the anticoagulant activity index of the vessel wall by 14.3% and an increase in vascular control over fibrinolysis, as judged by an increase in the index of fibrinolytic activity of the vascular wall by 13.5%. As a result of the use of aminosol, the activated partial thromboplastin time was inhibited by 16.5%, which was accompanied by a slowdown of the prothrombin time by 25.0% and an inhibition of the development of thrombin time by 9.4%.

Table. Hemostatic parameters in weakened newborn calves treated with aminosol

Indicators	Aminosol, n=34, M±m		Control, n=25, M±m
	exodus	after correction	
Platelet aggregation with ADP, s	25.2±0.12	36.7±0.09 p_1<0.01	41.4±0.07 p<0.01
Platelet aggregation with collagen, s	19.4±0.19	28.5±0.11 p_1<0.01	32.8±0.09 p<0.01
Platelet aggregation with thrombin, s	36.8±0.08	47.1±0.14 p_1<0.01	53.7±0.12 p<0.01
Platelet aggregation with ristomycin, s	47.2±0.10	32.7±0.17 p_1<0.01	39.4±0.12 p<0.01
Platelet aggregation with adrenaline, s	96.4±0.07	66.5±0.15 p_1<0.01	84.9±0.18 p<0.01
Vascular wall anti-aggregation index with ADP, units	1.34±0.10	1.58±0.06 p_1<0.01	1.65±0.14 p<0.01
Index of antiplatelet activity of the vascular wall with collagen, units	1.27±0.09	1.50±0.03 p_1<0.01	1.59±0.05 p<0.01
Index of antiplatelet activity of the vascular wall with thrombin, units	1.30±0.10	1.48±0.07 p_1<0.05	1.54±0.08 p<0.01
Index of antiaggregatory activity of the vascular wall	1.32±0.08	1.48±0.06 p_1<0.05	1.53±0.06 p<0.05
Index of antiplatelet activity of the vascular wall with adrenaline, units	1.35±0.05	1.57±0.04 p_1<0.05	1.66±0.05 p<0.01
Index anticoagulant activity vascular wall, units	1.12±0.06	1.28±0.07 p_1<0.05	1.34±0.04 p<0.01
Index fibrinolytic activity vascular wall, units	1.18±0.04	1.34±0.06 p_1<0.05	1.40±0.11 p<0.01
Activated partial thromboplastin time, s	27.9±0.28	32.5±0.30 p_1<0.01	39.9±0.27 p<0.01
Prothrombin time, s	12.8±0.22	16.0±0.29 p_1<0.01	17.6±0.26 p<0.01
Thrombin time, s	14.8±0.25	16.2±0.19 p_1<0.05	17.8±0.17 p<0.01

Legend: p - significance of differences in hemostatic parameters between the control group and the initial state of weakened calves, p1 - significance of the dynamics of hemostatic indicators during correction.

DISCUSSION

Optimal growth and development of calves at the very beginning of ontogenesis is associated with low activity of their hemostasis (Mal et al., 2018b) [13]. The onset of asthenization in the newborn phase can lead animals to the weakening of the functioning of internal organs (Tkacheva and Medvedev, 2020) with the development of disorders in the operation of the hemostasis system (Vorobyeva and Medvedev, 2020c). They are based on a decrease in the level of antioxidant properties of plasma, leading to an increase in concentrations of lipid peroxidation products in newborn calves born weakened, damaging the structure of blood plates, vascular walls and liver, leading to hemostasiopathy (Bespalov et al., 2018a). High AP activity in weakened newborn calves indicated excessive activation of receptor and intracellular platelet activation mechanisms. The acceleration of the development of AP in response to ristomycin in animals indicated an increase in their sensitivity to Willebrand factor under astenia conditions (Karpov et al., 2018). The rapid onset of AP with ADP in weakened calves was based on the enhancement in their blood plates of the conversion of arachidonic acid into an aggregate enhancing the effect of this inductor, thromboxane (Makhov and Medvedev, 2018a).

In weakened calves, reduced anti-aggregation activity of vascular endothelium was detected in the work. Without a doubt, this situation is ensured by the depression of synthesis in the walls of prostacycline vessels and nitrogen oxide. Another important component of vasopathy development in weakened calves is depression of synthesis in vessels of substances with anticoagulant and fibrinolytic activity - antithrombin III and tissue activator plasminogen (Boldov et al., 2018). The reduction of prothrombin time in physically weakened calves was a consequence of the activation of plasma hemostasis. Apparently, at its core, this has the appearance of excess active thromboplastin in their blood. The shortening of the activated partial thromboplastin time was based on an increase in the activity of the internal clotting mechanism (Oshurkova and Medvedev, 2018b). The acceleration of fibrin formation in weakened newborn calves was evidenced by the reduction of thrombin time.

The use of aminosol in weakened newborn calves has resulted in improvement of the overall condition of observed animals. Its use reduced the intensity of lipid peroxidation in the weakened young, reducing its stimulation to free circulation platelets. The found inhibition of AP development in weakened calves with respect to all inductors in case of aminosol use was largely due to the weakening of lipid peroxidation developing against this background, facilitation of platelet receptor and post-receptor mechanisms (Stepanova et al., 2018; Bespalov et al., 2018b). The elongation of AP development time in response to ristomycin found against the background of aminosol application indicated a decrease in the blood of weakened Willebrand factor calves, which is a cofactor of adhesion (Makhov and Medvedev, 2018b).

The use of aminosol in weakened calves stimulated the possibilities of anti-aggregation, anticoagulant and fibrinolytic capabilities of their vessels, which can be clearly explained (Medvedev and Kumova, 2007; Bikbulatova, 2018a). This was apparently provided by activation in its use of synthesis in the vascular endotheliocytes of these animals prostacycline, nitric oxide, antithrombin III and tissue plasminogen activator molecules. The delay in prothrombin time revealed in the weakened animals treated with aminosol indicated optimisation of plasma hemostasis mechanisms along the external route and was caused by physiologically necessary reduction of thromboplastin synthesis triggering clotting (Medvedev and Gamolina, 2008). The inhibition of the initially accelerated activated partial thromboplastin time detected after aminosol application indicated a decrease in activity of the internal hemocoagulation mechanism in calves. Development of this in combination with inhibition of fibrin formation detected by thrombin time dynamics was a manifestation of physiologically justified reduction of hemocoagulation (Bikbulatova, 2018b; Tkacheva, 2020).

CONCLUSION

Physically weakened newborn calves have low antioxidant blood protection leading to growth of lipid peroxidation products in it. These changes contribute to increased platelet activity, hemocoagulation and reduced hemostatic properties of vessels. Under conditions of aminosol application in weakened newborn calves, the level of antioxidant potential of plasma was increased, which led to decrease of lipid peroxidation products in their blood, decrease of platelet activity level and hemocoagulation with growth of functional properties of vascular walls. It is possible that the continuation of the use of this drug can lead to normalization of the taken-into-account indicators within a longer period of observation. It is planned to test this assumption in future studies.

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Optimization of Repeat Computed Tomography Simulation for a More Efficient Workflow in the Radiation Therapy Unit: A Single-Institution Experience

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ABSTRACT

The purpose of this study was to identify factors that increase efficiency, reduce performance rates, decrease overall patient waiting time, and maximize the degree of patient access associated with repeated computed tomography at the Radiation Oncology Unit of King Abdulaziz University Hospital. We retrospectively reviewed the records of all patients who underwent repeated computed tomography at the Radiation Therapy Unit of King Abdulaziz University Hospital from January 2014 to December 2018. Re-scanning was required for communication-related, clinical, patient-related, and technical issues. The characteristics of the study variables were defined using simple descriptive statistics. A chi-squared test was used to establish relationships between categorical variables. A p-value < 0.05 was considered statistically significant.During the study period, 241 cases were referred for re-scanning. The rate of re-scanning almost doubled from 2014 (3.2%) to 2018 (5.7%).

Clinical issues were the most common reasons for re-scanning (102 cases), followed by patient-related (89 cases), communication-related (45 cases), and technical issues (3 cases). The chi-squared test revealed significant associations among the variables (p-value=0.002, <0.001, and <0.001 for communication, clinical, and patient-related issues, respectively). We observed an increasing trend in the overall frequency of re-scanning. The number of re-scanning procedures due to clinical and communication-related issues decreased significantly in 2018, while those owing to patient-related issues increased significantly. Frequent planned evaluation of the workflow and identification of potential reversible and recurring issues responsible for decreased scanning efficiency will be beneficial in radiation oncology. Resolving such issues can improve the safety and quality of patient care..

KEY WORDS: RE-CT SCAN, WORKFLOW, RADIATION THERAPY, OPTIMIZATION.

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INTRODUCTION

The prolonged waiting time for the diagnosis and treatment of cancer is one of the most frequent concerns among attending clinicians and patients. This results to the disease being identified at a more advanced stage with worse prognoses. Longer wait times is not an empty issue, as this results to patient dissatisfaction which consequently has a critical effect on the healthcare outcomes for the patient (Elsaid et al., 2020). A recent study outlined the most common factors of longer wait times in an oncology department, which include waiting for lab results, team/clinic communication, patient factors, chemotherapy preparation, and chemotherapy consultation (Plourde et al., 2020). Their results pointed to laboratory turnaround times as the major cause of delay in oncology clinics.

Computed tomography (CT) scans are commonly used to visualize parts of the human body, in a more powerful way than regular x-ray procedures do. On a doctor's recommendation, a CT scan can be used to diagnose structural disorders, monitor treatments, visualize internal injuries, and more. In oncology departments, this procedure is used to detect, pinpoint and monitor tumors and infections to diagnose cancer and other diseases. A study conducted from 2015 to 2018 revealed that rapid access to CT scan procedures greatly reduced the waiting time during the diagnostic process (Franco Serrano et al., 2019). Even though repeat CT scans also cause longer waiting times for cancer patients, this procedure provides diagnostic clarity for health care specialists (Lovoli et al., 2019). Further studies must be conducted to evaluate the current state of Saudi healthcare system and its policies addressing the issue of inefficient workflow in hospitals.

In this study, the process required to ensure patient safety in accordance to the international and national accreditation parameters was evaluated, with the goal of improving the overall quality of service to cancer patients at the Radiation Therapy Unit in the institution. Additionally, this study aimed to identify potential opportunities that could increase efficiency, reduce performance rate, decrease the overall patient waiting times, and maximize the patient accessibility associated with re-CT.

MATERRIAL AND METHODS

In this study, we retrospectively reviewed the records of all re-CT cases at the Radiation Therapy Unit of our institution from January 2014 to December 2018. The reasons for re-CT were categorized into the following four groups: communication-related, clinical, patient-related, and technical issues. The Radiation Therapy Unit at our institution comprises more than 35 staff members, organized into the following four core disciplines: radiation oncology (RO), physiotherapy, nursing, and radiation therapy. It is the largest single-institution radiation facility in the country's western region and attends to 1100 new patients yearly, delivering more than 1200 courses of radiation treatment annually. Treatment planning for patients undergoing radiation treatment at this unit includes computed tomography simulation (CT-Sim). CT-Sim influences the initiation of radiation treatment for patients with cancer within an acceptable time duration, according to National Comprehensive Cancer Network guidelines.

The repeat CT (re-CT) procedures performed in the unit required auditing, to optimize the procedures used for tumors at different sites of the body, miscommunication issues, and clinical decisions, all of which eventually led to a re-CT appointment. Accordingly, a multidisciplinary team led by CT-Sim radiation therapists and physicians recently conducted a multifaceted audit of the current procedures and practices concerned with patient appointment for CT-Sim. Re-CT, which was performed because of an inefficient CT procedure, was found to be associated with increased cost. Statistical analysis was conducted using IBM SPSS version 23. Simple descriptive statistics such as counts and percentages were used to express the characteristics of the categorical and nominal variables, while continuous variables were presented as the mean and standard deviation. The chi-squared test was used to establish the relationship between the categorical variables. These tests were performed under the assumption of normal distribution. A conventional p-value of <0.05 was the criterion for rejecting the null hypothesis. The Hospital's research committee approved the study.

Table 1. Number of re-CT scan cases from January 2014 to December 2018

Variables	Year 2014	2015	2016	2017	2018	p-value
Total CT	885	975	1101	1206	1163	-
	0.03 ± 0.2	0.03 ± 0.2	0.05 ± 0.2	0.06 ± 0.2	0.06 ± 0.2	0.001a
Re-CT	28 (3.2%)	27 (2.8%)	52 (4.7%)	69 (5.7%)	65 (5.6%)	

aSignificance level set at 0.05 level using one-way analysis of variance. CT, computed tomography; re-CT, repeat CT

RESULTS AND DISCUSSION

A total of 241 cases were referred for re-CT between January 2014 and December 2018. Table 1 shows the percentage of re-CT cases per year. The number of re-CT cases noted were 28 (3.2%) in 2014, 27 (2.7%) in 2015, 52 (4.7%) in 2016, 69 (5.7%) in 2017, and 65 (5.6%) cases in 2018. The re-CT rate almost doubled from 3.1% in 2014 to 5.6% in 2018, showing a significantly increasing trend (p = 0.001) (Figure 1). Table 2 presents multiple comparisons of the number of re-CT scan cases per year. The result revealed significant differences in the number of such cases between 2014 and 2017, as well as between 2015 and 2017 and between 2015 and 2018. The collected data from patient charts survey revealed 4 major reasons for re-CT, which were divided into the following groups, Table 3: communication-related, clinical, patient-related, and technical issues. Four specific reasons were identified in the communication-related issues group.

Table 2. Multiple comparison of the number of re-CT cases across 5 years

(I) Year	(J) Year	Mean Difference (I-J)	95% Confidence Interval Lower Bound	Upper Bound	p-value
2014	2015	0.004	−0.018	0.025	0.987
	2016	−0.016	−0.039	0.008	0.378
	2017	−0.026*	−0.050	−0.001	0.034
	2018	−0.024	−0.049	0.000	0.053
2015	2016	−0.020	−0.042	0.003	0.127
	2017	−0.030*	−0.053	−0.006	0.005
	2018	−0.028*	−0.052	−0.005	0.009
2016	2017	−0.010	−0.035	0.015	0.818
	2018	−0.009	−0.034	0.017	0.884
2017	2018	0.001	−0.025	0.027	1.000

*The mean difference is significant at a level of 0.05. re-CT, repeat computed tomography

Thirty-five cases (14.5%) reported issues with the booking form, while a language barrier was not an issue in any case (0.0%). Ten cases (4.1%) experienced a mishap in the CT-Sim set-up. Clinical issues were also raised during the survey. Sixty patients (24.9%) reported that re-CT was performed because of the RO's decision (clinical): 54 (90%) of 60 patients underwent re-CT, because improvements in planning dictated that all patients with breast cancer had to undergo CT under an altered set-up to ensure better dosimetry quality, while the remaining 10% underwent re-CT owing to malfunctioning equipment. Four patients (2.3%) required re-CT, after a CT-Sim meeting decision (Sim meeting) (where all consultants met and discussed the patients' treatment plans), while 38 (15.8%) underwent re-CT for reasons related to planning and contouring, predominantly owing to difficulties in achieving dose constraints with three-dimensional (3D) planning and the need for a different set-up for volumetric intensity modulated radiotherapy (VMAT).

Table 3. Issues associated with re-CT

Variables		Count	%
Communication issues	Booking form	35	14.5
	Language barrier: RTT to patient	0	0.0
	CT Sim set-up error	10	4.1
Clinical issues	RO decision-Clinical	60	24.9
	Sim meeting decision	4	1.7
	Planning and contour	38	15.8
Patient-related issues	Preparation of the patient	18	7.5
	Patient-related delay	21	8.7
	Contrast related	4	1.7
	Anatomical variations	46	19.1
Technical issues	CT artifacts	3	1.2

RTT, radiation therapist; CT, computed tomography; re-CT, repeat CT; RO, radiation oncologist; Sim, simulation

Table 4. Major causes or issues associated with re-CT performance

Variables	Count	%
Communication issues	45	18.7
Clinical issues	102	42.3
Patient-related issues	89	36.9
Technical issues	3	1.2

re-CT, repeat computed tomography

Among the patient-related issues, 18 (7.5%) were related to the patients' self-preparation which means that the patient did not prepare well for the simulation Ct scan by emptying rectum or bladder or sometimes by filling bladder as indicated to them in the preparatory protocol; 21 (8.7%) were associated with patient-related delays, 4 (1.7%) were associated with contrast-related issues, and 46 (19.1%) were associated with anatomical variations. Eighty percent of these patients had tumors of the head and neck and required re-planning because of weight loss and ill-fitting masks. The only technical issues were associated with CT artifacts in 3 cases (1.2%). Based on the specific reasons described in this review, the RO's decision (clinical) was the most commonly cited reason (24%, n = 60) for re-CT, as shown in Figure 2. This was followed by cases with anatomical variations, and those with planning and contouring-related issues. The fourth most commonly noted reason was booking form-related problems, followed by patient-related delays, issues pertaining to patients' self-preparation, and those caused by CT-Sim set-up errors. As shown in Figure 1, language

barrier (radiation therapist to patient) was the least commonly observed reason for re-scanning. CT artifacts and contrast-related and SIM meeting decisions were other infrequently cited reasons (1.7% each) (n = 4).

As shown in Table 4 and Figure 3, clinical issues were the most commonly observed causes of re-scanning, followed by patient-related issues, communication-related issues, and finally, technical issues. Table 5 demonstrates the distributions of the three major causes of re-scanning from 2014 to 2018. Re-scanning owing to communication-related problems was observed in 9 (20.0%) cases in 2014, 8 (17.8%) in 2015, 19 (42.2%) in 2016, 6 (13.3%) in 2017, and 3 (6.7%) in 2018. Clinical-related reasons were reported in 4 (3.9%) cases in 2014, 4 (3.9%) in 2015, 15 (14.7%) in 2016, 57 (55.9%) in 2017, and 22 (21.6%) in 2018. Finally, patient-related issues were revealed to be responsible for re-CT in 15 cases (16.9%) each in 2014, 2015, and 2016, only 4 cases (4.5%) in 2017, and 40 (44.9%) in 2018. The association between these variables was evaluated using chi-squared tests, which revealed that the variables were significantly related to each other, with p-values =0.002, <0.001, and <0.001 for communication-related, clinical, and patient-related issues, respectively.

Table 5. Distribution of major issues associated with re-CT per year

Variables	Total	Year					p-value
		2014	2015	2016	2017	2018	
Total	241	28 (11.6%)	27 (11.2%)	52 (21.6%)	69 (28.6%)	65 (27.0%)	-
Communication issues	45	9 (20.0%)	8 (17.8%)	19 (42.2%)	6 (13.3%)	3 (6.7%)	<0.001a
Clinical issues	102	4 (3.9%)	4 (3.9%)	15 (14.7%)	57 (55.9%)	22 (21.6%)	<0.001a
Patient-related issues	89	15 (16.9%)	15 (16.9%)	15 (16.9%)	4 (4.5%)	40 (44.9%)	<0.001a
Technical issues	3	0 (0.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)	0.026a

[a]significance level set at < 0.05 using a chi-square test. re-CT, repeat computed tomography

Figure 1: Graphical representations of the number of re-CT cases from 2014 to 2018 Significance was set at p-value < 0.05. re-CT, repeat computed tomography

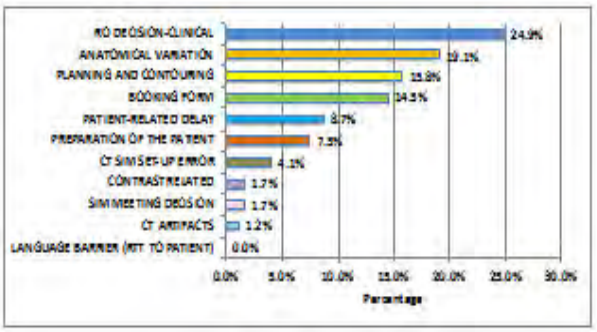
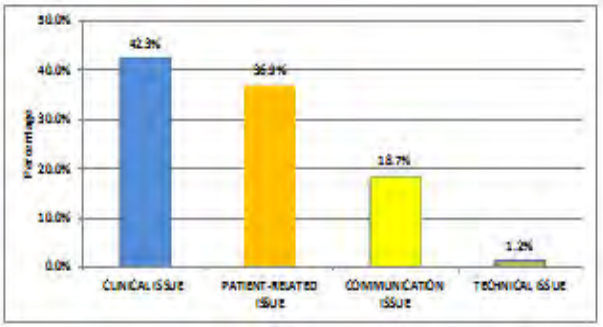


Figure 2: Issues associated with re-CT performance RO, radiation oncologist; SIM, simulation; CT, computed tomography; re-CT, repeat CT; RTT, radiation therapist



Re-scanning owing to clinical causes demonstrated an increasing trend between 2014 and 2017, accounting for 3.9% of all re-CT cases in 2014 and 2015, 14.7% in 2016, and 55.9% in 2017 (Figure 4). Nevertheless, a significant decrease was observed in 2018 (21.6%). The incidence rate of patient-related issues was almost consistent, accounting for 16.9% of all cases every year from 2014 to 2016, which subsequently decreased to 4.5% in 2017, before significantly rising in 2018 (44.9%). Among the 3 identified major causes of re-scanning, patient-related issues were the most commonly cited reasons from 2014 to 2015 and 2018, while communication-related issues were most common in 2016. The rate of re-CT owing to clinical issues was the highest in 2017.

DISCUSSION

In this study, an increasing trend in clinical, patient-related, and communication-related issues, which were major factors responsible for re-CT, were observed. The number of re-scanning procedures performed because of clinical and communication-related issues decreased significantly in 2018, while those performed because of patient-related issues increased significantly. Numerous demands are placed on patients with cancer and their families, which include coping with treatment schedules, the resulting side-effects, and adapting to lifestyle limitations and role changes (Merluzzi and Martinez Sanchez 1997). Cancer is associated with a substantial financial burden not just for the patient level, but also at a healthcare service and societal level (Barr et al., 2014). This is relevant to the current need to rationalize the expenditure of resources in a specialty with rising

treatment costs, and the decisions made can have a significant impact on the patient's quality of life (Pearce et al., 2001).

There is a relevant study conducted by Soo et al. (2019) which analyzed patient experiences and perspectives before, during and after imaging-guided breast biopsies. They also proposed policies and strategies to overcome hurdles in optimizing the patient experience. Results showed that exhibiting compassion in delivering cancer diagnoses, along with optimizing the physician-patient communication and developing a patient-centered approach all contribute to overall general satisfaction of the patient population. Additionally, they stated that long wait times in the context of definitive diagnoses are often "intense and agonizing" experiences and contributes to the stress and anxiety experienced by the patient.

Figure 3: Major causes of or issues related to re-CT scanning of patients re-CT, repeat computed tomography

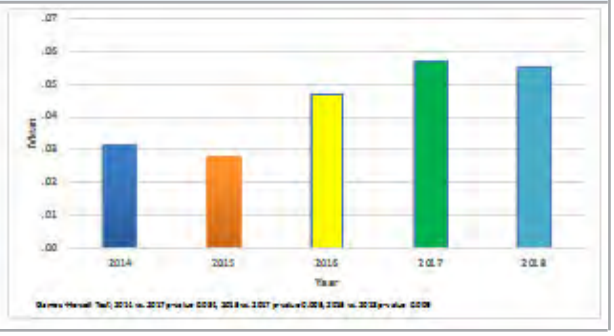
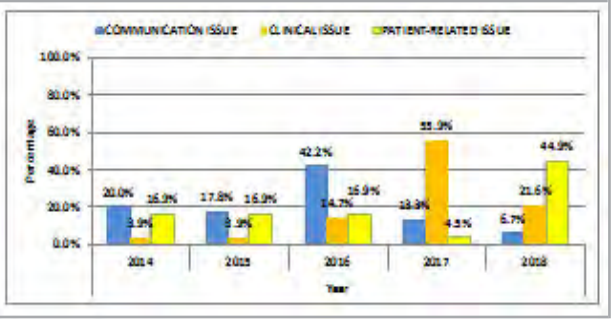


Figure 4: Distribution of major issues of re-CT per year re-CT, repeat computed tomography



On a similar study, where oncology patients' experiences of a surveillance CT were reviewed (Raaschou et al., 2019). Results showed that radiographers' focus on the technological aspect of clinical management instead of establishing relations and showing sincere interest and empathy, contribute to the "undesirable" experiences most subject patients share. The study highlighted that establishing communication and relationship with the patient is as important as providing quality radiological interpretation and diagnoses.A literature review conducted by Pearce et al. (2001) in the United Kingdom revealed that the costs associated with cancer treatment and care were complex. These costs included those related to cancer treatment and service delivery, indirect costs to patients and their caregivers, and the human cost of cancer and its treatment, such as deterioration in the quality of life, lifestyle and role changes, symptom-related distress, and wastage of time.

They identified a lack of research on the development of cost-effective healthcare services. With the escalating cost of healthcare and the economic recession, healthcare institutions in Saudi Arabia have identified the need to re-evaluate and reduce expenditures without compromising on the quality of patient care. For instance, CT is a commonly used, less expensive imaging modality, which is instrumental in facilitating the diagnosis of a wide range of medical conditions, especially cancer (Jabali et al., 2015). Ultimately, this study aimed to identify opportunities to increase the efficiency of CT, to reduce the rate of re-CT and patient waiting time, and maximize patient access to this essential diagnostic modality, as a part of treatment. We discovered that clinical problems such as the RO's decision to repeat the scan, because of mismatch between set-up and planning requirements; planning and contouring-related issues; anatomical variations (patient-related); and booking form-related issues (communication-related issue) were the most common causes of re-CT from 2014 to 2018. These findings are vital, as they indicate the need for providing continuous education to clinical teams on the services they are expected to provide, as the burden of cancer is already enormous on patients and their families.

During the study duration (2014–2018), the rate of re-CT almost doubled from 3.1% in 2014 to 5.7% in 2018, thus, showing a prominent increasing trend. According to the findings, the RO's decision (clinical) was the most commonly cited cause of re-CT, which accounted for 60 of 241 cases from 2014–2018. Overall, 90% of the 60 cases underwent re-scanning because of changes in the breast cancer set-up for the achievement of proper constraints; 10% (6 cases) of the cases underwent re-CT because of malfunctioning equipment.The deep inspiration breath-hold technique (DIBH) was not utilized at our center, and switching to VMAT planning was the only way to improve coverage whilst respecting normal tissue constraints for patients with breast cancer. Moreover, differences in the CT protocol around the world must be taken into account, especially those pertaining to the inclusion of the internal mammary lymph (IM) nodes (Duane et al., 2019), which may be more challenging with 3D planning (explaining the more frequent need to switch to VMAT). The observed surge in the re-CT rate could be related, at least partially, to the appointment of a few new ROs with different practice backgrounds with respect to IM radiotherapy, and the use of intensity-modulated radiotherapy (IMRT) for different sites in general. For technical reasons related to differences in the machines used at our institution, the switch from 3D to VMAT for breast cancer-related radiotherapy requires a different set-up, resulting in re-CT. Differences in the set-up for 3D and VMAT are also applicable to other sites, e.g., bladder emptiness and fullness for pelvic IMRT.

Some measures can be taken to attempt to reduce the re-CT rate, improve CT efficiency, and decrease the degree of patient anxiety related to unplanned treatment delays in the above-mentioned situations. First, certain screening criteria must be implemented to aid in the identification of patients who are likely to benefit from DIBH or VMAT, followed by re-scanning with a different set-up but at the same appointment (Czeremszynska et al., 2017; Rice et al., 2017). Second, institutional practice guidelines should be developed, to decrease the frequency of variations in practice, to limit the need for re-CT associated with unpredictable anatomical factors that complicate planning and dictate technique changes. Third, while the timely identification of such patient-related factors may not change the need for re-CT, it can shorten delays in treatment initiation and their subsequent effects on treatment outcomes (Khorana et al., 2019), thereby alleviating the degree of anxiety and distress. Finally, reducing the inevitably high demand-related machine malfunction rate through rigorous quality assurance and proper maintenance programs can lower the magnitude of the problem and the associated mistrust from the patient's side and reduce the worsened disease outcomes related to the tumor repopulation effect (Bohmer and Edmondson 2001; Fiol and Lyles 1985).

In 2019, a study by Beaumont et al. compared the standard radiological workflow and a novel "hybrid" workflow they proposed. Their study pointed to trial nonconformities like blank reports, unsigned reports, missing/wrong patients' appointment dates as unnecessary errors that consume valuable time. Their proposed hybrid workflow saved around 87% of radiologists time and could offer a plausible opportunity for reducing costs with improved imaging quality. Overall, they pointed out that time efficiency in radiological clinical trials can be improved; electronic case report forms reduces nonformities; and that radiologists can delegate non-essential tasks. Al Hroub et al. (2019) also conducted a similar study where they made use of lean thinking concepts and tools to improve the workflow efficiency in their outpatient oncology center. After implementation, the mean clinic waiting time decreased from 72.5 minutes to 19.5 minutes and 21 minutes at two different quarters (period of implementation). They attributed this to the redesigned electronic appointment system which reduced patient waiting time, improved patient satisfaction and resource utilization. Additionally, their updated workflow reallocates health-care providers' time and promotes a new perspective towards a direct and individualized patient care.

Anatomical variation was the second most commonly cited reason for re-CT, including weight loss or gain during radiotherapy. Forty-six cases of anatomical variation (patient-related) were identified; the majority was observed in patients with head and neck cancer. Other researchers have also identified that these sites are among the most common sites requiring re-scanning (Carroll and Edmondson 2002). The optimization of nutritional support and availability of an onsite dietitian, an aspect that is being investigated, would theoretically decrease the magnitude of weight loss and need for re-scanning (Colasanto et al., 2005). Interestingly, there is a lack of evidence supporting the efficiency of prophylactic feeding for weight loss prevention and subsequent re-planning (Brown and Yabroff 2006). However, adaptive radiotherapy for head and neck cancers improves the coverage of shrinking tumors and reduces the degree of overdose to the organs at risk (Surucu et al., 2017).

Our findings revealed a statistically significant association between the year and cause of re-scanning. A notable increasing trend was observed from 2014 to 2017 in the re-scanning rates associated with clinical issues that coincided with the enrollment of new ROs. Moreover, re-CT rates caused by patient-related issues have increased significantly from 2017 (4 cases) to 2018 (40 cases); consequently, attention to patients' preparation strategies, including proper education to ensure satisfactory planning and treatment delivery, is of paramount importance. The use of written and audiovisual material may improve patient satisfaction and more importantly, education outcomes (Saeed 2018; Savage et al., 2017).

Communication-related issues, especially booking form-related issues, were identified to be among the most commonly cited causes of re-CT. Two very important measures that can minimize the degree of these issues are already under implementation. First, the development and frequent updating of departmental policies and procedures are crucial for the maintenance of the program's quality and will decrease the amount of information to be filled in the booking form, thereby reducing error rates, and ultimately, re-CT rates. Second, shifting to paperless forms from paper-based booking ones and the development of standard site and disease-specific care plans may also improve the quality and efficiency of booking forms, and subsequently, reduce the rate of re-CT. A study conducted by Woolen et al. (2018) attempted to utilize and asses the efficacy of online patient portals, specifically in relation to the time of release of CT scan results. Their research revealed that the outpatient population prefer to receive imaging results regarding a probable cancer diagnosis as soon as possible with direct communication with their physician over the telephone, as compared to receiving the result in their physician's office or over other media such as electronic mail. However, they surveyed only their local outpatient population, and may not apply to other departments or their inpatient population. Further studies may be conducted if this approach overcomes other bureaucratical or clinical hindrances to improve workflow efficiency.

Healthcare institutions play a major role in the diagnosis, treatment, and monitoring of patients with cancer. Therefore, it is important that they initiate the development of processes that involve procuring new knowledge and the application of this knowledge (Fiol and Lyles 1985). Healthcare delivery researchers state that learning is a cyclical and multi-level process (Bohmer and Edmondson 2001; Carroll and Edmondson 2002;

Hamel et al., 2014). System errors and inadequacies are frequently organizational, including multiple individuals and/or systems within the hospital, and necessitate a more holistic view of the organizational and clinical characteristics of care (Goodman et al., 2011; Hamel et al. 2014; Ramanujam and Rousseau 2006).

The present study is the first to identify opportunities that increase efficiency, reduced performance rate and overall patient wait times, and maximized patients' access to re-CT, as part of radiation therapy in Saudi Arabia. It is limited to the local population of the Radiology Oncology Unit of King Abdulaziz University Hospital. Lastly, this study may be used as a guide to reform and redesign policies accordingly to improve overall patient satisfaction and produce an efficient workflow beneficial to both healthcare workers and patients.

CONCLUSION

While the need for re-CT is inevitable in any radiation therapy unit, frequent planned evaluation of the workflow and the potential reversibility and recurrence of issues responsible for decreased CT efficiency in any department is essential, particularly at times when the degree of the changes in manpower is significant. The resolution of these recurring issues may aid in reducing the rate of re-CT, and inevitably improve the safety and quality of patient care.

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Conserved Markers Order in Quantitative Trait Loci Confer Resistance Against Black Root Rot Disease in Cotton, (*Gossypium*)

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ABSTRACT

Black root rot disease is documented for substantial reducing cotton yield and fiber quality. The isolation of candidate resistant genes in tetraploid genome AADD cotton species (2n=4x=52) remains challenging in the absence of research of black root rot resistance on progenitor DD genome diploid cotton, *Gossypium* spp. In this study, by exploiting Phytozome database, a comparative map of the black root rot-resistance quantitative trait loci in DD genome was constructed. Simple sequence repeats markers associated with these three quantitative trait loci in the AA genome were used as "anchored-probes" frameworks for establishing relationships between the two cotton genomes AA and DD. Present findings showed that there was conserved orders among mapped simple sequence repeats markers on AA genome and the physical map of these simple sequence repeats markers on DD genome. It is suggested that the syntenic loci on chromosome 2, 7 and 11 on DD genome could correspond the black root rot resistance in cotton, *Gossypium* spp. This study could serve as a fundamental step in isolating and introducing the resistance gene against black root rot into elite cotton cultivars.

KEY WORDS: COMPARATIVE MAPPING, RESISTANCE GENE, PHYTOZOME, SIMPLE SEQUENCE REPEATS, QUANTITATIVE TRAIT LOCI.

INTRODUCTION

Diseases exhibit an adverse impact on cotton (*Gossypium* spp.) production. The yield loss is projected at approximately 60% of the annual potential production (Blasingame, 2005; Rothrock, 1997). Black root rot (BRR) is a seedling disease caused by *Thielaviopsis basicola*, a soil-borne pathogen fungal with a broad infection spectrum of crops. Since its first reported case on cotton in Arizona in 1922 (King and Presley, 1942), it has become one of the significant threats in cotton industry. Because of the susceptibility to BRR of the two commercially important tetraploid cotton genome AADD species *G. barbadense* and *G. hirsutum*, tremendous efforts have been made toward developing BRR resistance germplasm. However, BRR partial resistance has only been demonstrated in several studies conducted in uncommercial AA genome specie *G. arboretum* (variance PI1415) and *G. herbaceum* (variance A20) (Wheeler et al., 1999; Wheeler et al., 2000 Khan et al., 2016).

Most recently, by employing crossbreeding from these two cultivars, followed by genetic analysis with simple sequence repeats (SSR) markers, three quantitative trait loci (QTL) BRR5.1, BRR9.1, BRR13.1 were demonstrated to improve BRR resistance (Niu et al., 2008). Nevertheless, there is a limited number of reports on how cotton DD genome specie, which is the progenitor of the cotton

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genome AADD species, confer BRR tolerance. The importance of comparative mapping is the establishment of the syntenic relationships between genomes from different species (Kliebenstein et al., 2001; Murphy et al., 2001; Schmidt, 2002). Mountain of evidences have accumulated in comparative mapping analysis in many species of great economic importance, such as *Pinaceae*, soybean (*Glycine max*), barrel medic (*Medicago truncatula*), cabbage (*Brassica oleracea*), potato (*Solanum tuberosum*), and *Arabidopsis thaliana* (Babula et al., 2003; Gebhardt et al., 2003; Grant et al., 2000; Lukens et al., 2003; Zhu et al., 2003, Kirungu et al 2018).

By using a standard set of frequently applied markers such as SSR and RFLP, comparative mapping assists the translation and transferring the information from one genomic map to another, such as verification of QTL, obtaining better knowledge of genome evolution, and identification of candidate genes underlying QTL (Duran et al., 2009 Kirungu et al., 2018). Specifically, the idea of transferring map information to improve disease resistance has been conducted in coffee (*Psilanthus*). Molecular markers were used to isolate the new resistance genes which were subsequently introduced novel more robust sources into commercially elite coffee varieties (Hendre et al., 2011 Kirungu et al., 2018).

The purpose of this study is to physically map the published SSRs from three QTL conferring BRR resistance on AA genome to DD gemome in cotton. By utilizing CottonGen and Phytozome database, our findings suggested that there was a correlation between the genetic map in AA genome and physical map in DD genome. A comparative map was constructed, illustrating the conserved order of SSR markers from the genetic mapping results in diploid AA genome from Niu et al. (2008) (Niu et al., 2008) and in DD genome. These results will shed new lights in understanding of shared synteny of QTL conferring black root rot disease between two diploid genomes in cotton, which could also indicate that DD genome possibly harbor R genes for BRR resistance.

MATERIAL AND METHODS

The study was carried out at Department of Plant and Soil Science, Texas Tech University, USA from January 2014 to May 2014.

CottonGen: CottonGen is an online mapping database for cotton (Yu et al., 2014). Cotton Gen contains information on genomic, genetics, breeding, and molecular genetic markers. It also incorporates genomic sequences of different cotton genomes, markers, and traits. Additionally, various platform such as BLAST, JBrowse, MapViewer, Primer3 are also included in the website.

Retrieving AA genome-derived SSR markers sequence: Visited the CottonGen website (https://www.cottongen.org/). Along the Tools Quick Start, followed 'Search Markers' (Figure 1).In the 'Marker Name' section, clicked on 'contains' in the first box and then typed the name of the marker in the second box (Figure 2). Used the marker name in the publication of Niu et al. (2008), page 1318, Figure 3.In the 'Marker Type' section, clicked on 'SSR'. Then hit 'Search'.In the resulting search table, clicked any of the records that are showed in the table. In the 'Marker Overview', clicked on the 'Source Sequence' to get the sequence of the markers (Figure 3). Copied the sequence of the marker in Notepad program of Microsoft Windows.

Figure 1: The CottonGen website entry display

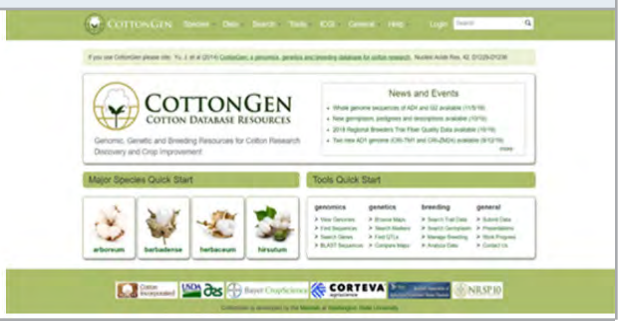


Figure 2: CottonGen SSR marker entry display

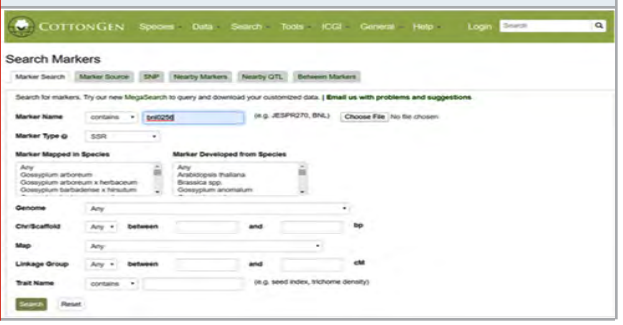
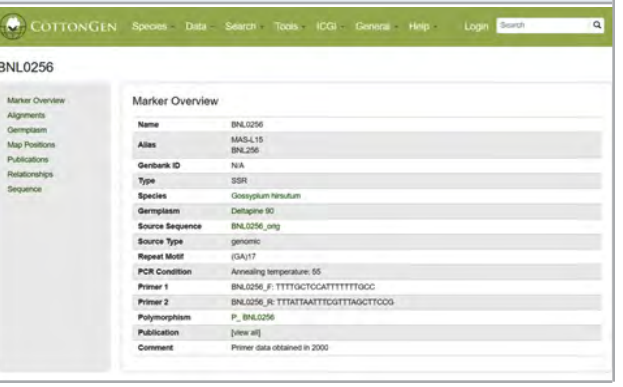


Figure 3: CottonGen representation of the selected SSR marker



Phytozome: Since its development from 2008, Phytozome has become a connective platform for many research on plant genome. Besides its easily and friendly accessible database, which contains 25 plant genomes including cotton, Phytozome is also equipped with tools for comparative analysis so that scientists can compare every plant genes at the various level of sequences (Goodstein et al., 2012). Localizing AA genome-derived SSR markers

to DD genome: Visited the Phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html#). Along the top menu header, visited 'Species' and chose '*Gossypium raimondii* v2.1' (Figure 4).

Figure 4: Phytozome website entry display

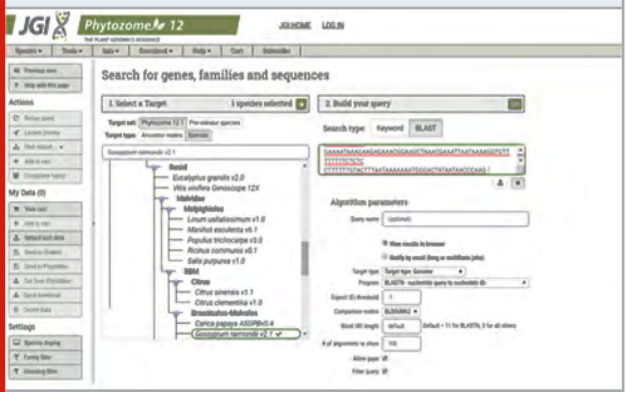
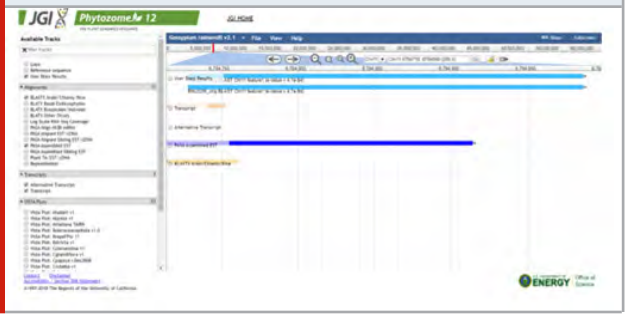


Figure 5: Phytozome representation of BLAST results

Figure 6: Phytozome representation of chromosomal position of SSR marker



In the new resulting page, along the menu under the title '*Gossypium raimondii* v2.1 (Cotton), clicked on 'BLAST search' (Figure 5).In the second column '2. Build your query', pasted the copied marker's sequence into the box the says 'Entered a single sequence...'. Then hit 'Go'. The BLAST results page showed the most significant hits. Selected the first hit with the darkest color arrow bar. In the 'Target View' section, clicked on that arrow bar in the 'Feature scale" column. In the close-up viewing mode in JBrowse, copied the information of the chromosome in the first box and the physical position of the marker in that chromosome in the second box (Figure 6).

RESULTS AND DISCUSSION

It has been showed here that after anchoring the SSR markers from the results of Niu et al. (2008) on DD genome, there was collinearity between the genetic map of SSR markers associated three QTL conferring BRR on AA genome and the physical position of these SSR markers on DD genome (Table 1). We still observed some minor SRR markers inversions, especially in the chromosomal regions on DD genome which corresponds to the linkage group A9. The same observation was also portrayed in study by Rong et al. (2004). These inversions could be explained by the rearrangement of the chromosomal segments during evolution of AA and DD genomes after separating from the first common ancestor (Rong et al., 2004). One more explanation could be the order of SSR markers were calculated based on the recombination frequency which could be utilized to measure the genetic distance between two loci, whereas the physical map was based on the number of nucleotides between two loci (O'Rourke, 2014). Overall, this result confirmed the accuracy of the genetic map in previous study by Niu et al. (2008).

Evolutionary evidence has suggested that from the origin of a common ancestor, diploid cotton species continued evolving and subsequently dividing into eight current monophyletic groups denoted as A–G, and K. A hybridization occurred approximately 1 to 2 million years ago between two diploid cotton species (2n = 2x = 26): *G. raimondii* (D5) and *G. arboreum* (A2) or *G. herbaceum* (A1). This event introduced the emergence of allotetraploid species (2n = 4x = 52) (Wendel, 1989; Wendel et al., 1995). After undergoing the polyploidization and following independent evolution processes, these tetraploid cottons differentiate into six present tetraploid species including *G. hirsutum* (AD)1, *G. barbadense* (AD)2, *G.tomentosum* (AD)3, *G. mustelinum* (AD)4, *G. darwinii* (AD)5 and *G. ekmanianum* (AD)6 (Grover et al., 2015).

Thanks to its information, versatility and easy detection in genetic experiments, SSR markers have been widely employed in QTL mapping and saturating in many plant genomes (Blenda et al., 2006; Khan et al., 2016). In cotton, mountain of evidence has been gathered in data mining to discover and characterize new SSR marker to narrow down the QTL regions. The ultimate purpose of this process is to isolate the candidate genes responsible for desired agricultural traits, including disease tolerance (Blenda et al., 2006; Kirungu et al., 2018; Tabbasam et al., 2014; Yu et al., 2012; Yu et al., 2011). However, the susceptibility to BRR of two commercial tetraploid cotton species *Gossypium hirsutum, Gossypium barbadense* or crosses generated from these two species with other tetraploid species have hindered the development cotton cultivars conferring resistance to this disease. As a result, researches mainly focused on elaborating how cotton diploid genomes contribute to improving BRR resistance.

In this study a Phytozome-based comparative mapping between two cotton diploid genomes revealing conserved markers order in quantitative trait loci conferring resistance against black root rot disease has been demonstrated. We report here a new method that could physically map AA genome-SSR markers in D genome by using Phytozome database. Given the collinearity between regions of AA and DD genomes in this study, we suggested that the syntenic regions on DD genome could also confer the BRR resistance. These regions were on chromosome 2 from position 7879824 to position 59691832, chromosome 7 from position 3320225 to position 58206145, chromosome 11 from position 5509175 to position 57112593. More research should be done to increase the density of SRR markers in these regions to isolate candidate R-genes.

Table 1. Correlation between the genetic map on AA genome and physical map on DD genome of SSR markers.

Linkage group number	SSR markers on A genome (appear in order)	Hypothetical synteny order on D genome	SSR markers on D genome (appear in order)	Chromosome number on D genome	First position on D genome	Last position on D genome
LGA9	NAU0921	MGHES27	MGHES27	11	5509175	5509752
	MGHES41	TMA18	BNL0256		6784715	6784998
	BNL3895	BNL0256	NAU1041		9892984	9898779
	NAU1041	NAU1041	MGHES41		11011950	11014308
	BNL0256	BNL3895	NAU0921		17797360	17798057
	TMA18	MGHES41	BNL3895		23404926	23405317
	MGHES27	NAU0921	TMA18		57111815	57112593
LGA13	BNL3442	BNL3442	BNL3442	7	3320225	3320674
	BNL1034	BNL1034	BNL1034		5461060	5461360
	NAU0760	NAU0760	NAU0760		6856140	6856464
	BNL2589	BNL2589	BNL2589		6986385	6986892
	BNL3147	BNL3147	BNL3147		7340897	7341396
	BNL1681	BNL1681	BNL1681		14808675	14808963
	BNL4094	BNL4094	BNL4094		19878990	19879398
	BNL2632	BNL2632	BNL2632		24142720	24143238
	NAU1063	NAU1063	BNL0625		28319473	28319761
	BNL0625	BNL0625	NAU1063		36248258	36250575
	BNL1408	BNL1408	BNL1408		43869105	43869532
	BNL1066	BNL1066	BNL0836		52098774	52099213
	BNL1231	BNL1231	BNL1066		54550357	54551604
	MGHES16	MGHES16	BNL1231		57124813	57125015
	CIR196	CIR196	MGHES16		58185366	58186970
	BNL0836	BNL0836	CIR196		58205755	58206145
LGA5	BNL1683	CIR114	BNL3580	2	7879824	7880305
	MGHES10	BNL3580	CIR241		7879830	7880229
	BNL2646	CIR241	CIR114		8053239	8053760
	BNL3791	BNL1667	BNL1667		9774273	9774659
	CIR049	BNL3888	BNL3888		11188791	11189262
	CIR089	BNL3090	BNL3090		13608472	13608924
	BNL3090	CIR089	CIR089		16149735	16149932
	BNL3888	CIR049	CIR049		16247068	16247503
	BNL1667	BNL3791	MGHES10		24401561	24403232
	CIR241	BNL2646	BNL3791		32038546	32038941
	BNL3580	MGHES10	BNL2646		43358571	43358990
	CIR114	BNL1683	BNL1693		59691568	59691832

CONCLUSION

Conclusively, our study revealed that three QTL regions conferring BRR resistance in AA genome exhibited a significant collinearity with DD genome. While the orders of SSR markers on linkage group A13 on AA genome and on its counterpart region of DD genome are conserved, there were some minor inversions among of SSR markers on linkage group A7 and A5 on two genomic regions that could be explicable by the rearrangement of the chromosomal segments or recombination frequency. The results from this paper could be further used for fine

mapping resistance genes against BRR in DD genome in the future.

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Association Between Short Sleep Duration and Childhood Obesity in School-Going Children: A Mini Review

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ABSTRACT

Sleep is a physiological process that fulfils an important homeostatic function. Short sleep duration (SSD) is defined as sleep that is substantially shorter than the optimal duration of 8-hours for adolescents and adults (18 years and above), and 9-hours for children (3-17 years). Insufficient sleep is associated with adverse health outcomes such as obesity, type 2 diabetes, stroke, hypertension, coronary heart disease, and other chronic kidney diseases. Globally, 30% of the children with obesity have been reported to experience sleep disorders. The present review has been aimed to describe the association between SSD and weight gain during childhood using a sample of school-going children (5–17 years). Specifically, based on a systematic meta-analysis of original research articles, it aimed to assess the impact of SSD on school-going children. Findings showed that SSD is a risk factor for children aged 5–17 years who experience insufficient sleep (< 9 hours/day). Epidemiological studies such as case-control, observational, follow-up, and meta-analysis studies confirm SSD as a risk factor for the development of obesity and other diseases through various mechanisms. Thus, based on numerous studies on SSD in children, it is confirmed as a risk factor for childhood obesity. It is therefore recommended to prevent SSD to avoid future complications.

KEY WORDS: SHORT SLEEP DURATION, OBESITY, RISK-FACTORS, CHILDHOOD OBESITY.

INTRODUCTION

Sleep is defined as the resting state of expected insentience from which any individual can be aroused, (Ezenwanne and research, 2011). It is a multi-faceted and vital physiological mechanism that is assessed using regular sleep logs, actigraphy, and polysomnography. In children, insufficient sleep has been documented to have deleterious effects on health, and sleep duration has been found to be associated with non-communicable diseases (Brazendale et al., 2019). Sleep is considered as food for the brain, as important brain functions occur during sleep, therefore, skipping sleep can be harmful and deadly as well, (Roberts et al., 2020).

The present review has been aimed to describe the association between Short Sleep Duration (SSD) and weight gain during childhood using a sample of school-going children (5–17 years). Specifically, based on a systematic meta-analysis of original research articles, it aimed to assess the impact of SSD on school-going children. Findings showed that SSD is a risk factor for children aged 5–17 years who experience insufficient sleep (<9 hours/day). Epidemiological studies such as case-control, observational, follow-up, and meta-analysis studies confirm SSD as a risk factor for the development of obesity and other diseases through various mechanisms. Thus, based on numerous studies on SSD in children, it is confirmed as a risk factor for childhood obesity. It is therefore recommended to prevent SSD to avoid future

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complications. Sufficient sleep duration contributes to the regulation of hormonal and metabolic processes in children (0–17 Years) (Miller et al., 2015).

According to the National Sleep Foundation (NSF) in the US, the minimum required sleep duration is 14-17 hours for new-borns (aged 0-3 months), 12-15 hours for infants (aged 4-11 months), 11-14 hours for toddlers (aged 1-2 years), 10-13 hours for preschool children (aged 3-5 years), 9-11 hours for school-aged children (aged 6-13 years), 8-10 hours for adolescents (aged 14-17 years), 7-9 hours for young and middle adults (aged 18-25 years and 26-64 years, respectively), and 7-8 hours for older adults (aged over ≥ 65 years) (Foundation, 2019). Similar recommendations have been adopted in Canada (Chaput et al., 2016). Similarly, the American Academy of Sleep Medicine (AASM) and Sleep Research Society recommend that adults require 7–9 hours of sleep (Watson et al., 2015, Hirshkowitz et al., 2015, Tobaldini et al., 2019). The sleep requirements for children recommended by AASM have been presented in Figure 1. is defined as a total sleep duration of less than 6 hours/day. It is often identified using polysomnography on a single night. Inadequate sleep is defined as sleep duration that is lesser than age-based recommendations, and it is commonly present in school-going children, (Tambalis et al., 2018).

Figure 1: Showing suggested amount of sleep for pediatric population

Age	Recommended Sleep Hours per 24 Hour Period
Infants: 4 to 12 months	12 to 16 hours (including naps)
Toddlers: 1 to 2 years	11 to 14 hours (including naps)
Preschoolers: 3 to 5 years	10 to 13 hours (including naps)
Gradeschoolers: 6 to 12 years	9 to 12 hours
Teens: 13 to 18 years	8 to 10 hours

*The American Academy of Pediatrics (AAP) has issued a Statement of Endorsement supporting these guidelines from the American Academy of Sleep Medicine (AASM).

Specifically, in children aged 5–16 years, the pressure of extra-curricular activities, disturbances from electronics, noisy environment, and home-work or academic tasks are considered as risk factors for SSD (Glaser and Styne, 2017). In the US, the mean sleep duration for children was 7.1 hours, with 29.2% children sleeping for less than 6 hours. The corresponding figure from Canada and the UK were 11.3% and 9.8% (Itani et al., 2017). The underlying cause of SSD could be sleep disorders, which is a large group of diseases with a nominal incidence in the general population. Insomnia, a well-documented and common sleep problem, is defined as a sleep disorder that is characterized by complications in sustaining sleep. Further, it is associated with impaired daytime functioning. Insufficient sleep often develops into more serious health issues such as insomnia and other sleep disorders. Additionally, SSD is associated with increased incidence of cardiovascular diseases (CVD), hypertension (HTN), and arrhythmias (Tobaldini et al., 2019).

Sleep duration has been found to be associated with innumerable health issues such as chronic diabetes, kidney problems, CVD, and obesity (Rosinger et al., 2018). On the other hand, obesity predicts health issues such as diabetes, CVD, early mortality, HTN, and lower quality of life (Buxton et al., 2010). Previous systematic reviews have confirmed that SSD is associated with major health issues such as mortality, HTN, CVD, stroke, diabetes, obesity, metabolic abnormalities, and atherosclerosis (Gallicchio and Kalesan, 2009, Wu et al., 2014, Leng et al., 2015, Itani et al., 2017). Grandner et al defined SSD as sleep duration of < 6 hours or 1/4th of the day of sleep deficiency.

SSD is associated with medical complications at a later age. Further, overweight in young children is associated with obesity-related conditions such as asthma, HTN, hyperlipidaemia, type 2 diabetes mellitus (T2DM), and higher morbidity and mortality in adulthood, (Taveras et al., 2008). SSD may also lead to psychiatric disorders and cardiometabolic risk factors such as poor glucose homeostasis and dyslipidaemia (Lombardero et al., 2019). Poor sleep in children (aged 0–17 years) puts them at a higher risk for weight gain, which could lead to overweight or obesity. In this context, a meta-analysis of epidemiological and cohort data revealed an unintentional relationship between SSD and health issues (Jike et al., 2018).

Inadequate sleep and childhood obesity: The term overweight and obesity are defined as accumulation of additional fat in the body. Obesity is identified using a screening tool known as Body Mass Index (BMI), which is calculated as weight in kilograms divided by the square of height in meters (Khan et al., 2019). A that of 25.0 kg/m2–29.9 kg/m2 is considered as overweight, and that of 35 kg/m^2–40 kg/m^2 or higher is considered as morbid obesity (Khan et al., 2014). Weight gain in children is connected with short and long-term adverse outcomes. In children, the occurrence of childhood obesity has increased due to an increase in the prevalence of SSD (Ash and Taveras, 2017).

Presence of excess body fat in children is known as childhood obesity. Among the common health issues that have been observed in the 21st century, childhood obesity is defined as proning the disease since 2016, with more than 124 million children aged 5–19 years being identified as obese and 213 million children being identified as overweight. Childhood obesity is connected with psychological problems; lower educational attainment; and harmful co-morbidities later in life, such as dyslipidaemia, T2DM, non-alcoholic fatty liver disease (NAFLD), CHD, and HTN (Spinelli et al., 2019). Further, musculoskeletal disorders are one of the undocumented complications associated with obesity and SSD (Al Shehri et al., 2013).

Childhood obesity and severe obesity put individuals at risk of CVD, obstructive sleep apnoea, impaired glucose tolerance, increased exposure to bullying, and NAFLD (Carsley et al., 2019). Globally, childhood obesity

has become a major health concern. The increase in childhood obesity has been linked to the simultaneous increase in the tendency to experience the metabolic syndrome (MetS). The future complications of obesity are T2DM, HTN, dyslipidaemia, and MetS. However, cardiometabolic risk factors are considered to be modifiable. Sleep plays a major role in the growth and development of children through its control of the diurnal rhythm that is connected with energy homeostasis, (Seo and Shim, 2019).

The incidence of childhood obesity has tripled in the past decades, leading it to be recognized as a global health threat. It is associated with numerous adverse outcomes such as poor academic performance, psychological conditions, cardiovascular disease, and permanent obesity. The common causes of childhood obesity are sedentary lifestyle with high-calorie eating habits (uncontrolled diet) (Li et al., 2017b). Further, preceding epidemiological studies have confirmed that the increase in the prevalence of obesity is associated with the decrease in sleep duration in children (Kelishadi, 2007, Li et al., 2011). Some previous studies have also documented that SSD during childhood is connected with cardiometabolic risk factors (Chaput et al., 2006, Li et al., 2017a, Touchette et al., 2008). Recently, the global prevalence rate of childhood obesity was estimated to be 6.7%, and it is expected to reach 9.1% by 2020 (Bin-Hasan et al., 2018).

Afshin et al., 2017) reported that 107.7 million children were obese, with a global prevalence of 5%. The Canadian national government prioritized the implementation of efforts for prevent and manage weight gain (obesity) in children (Carsley et al., 2019). Countries in the Gulf Cooperation Council (GCC) have documented the highest prevalence of T2DM and obesity, and surveillance of childhood obesity is considered central to tackling this obesity epidemic (Al Hammadi and Reilly, 2019).

Statistically, the age-based BMI classification for children defines overweight as the 85th–95th percentile, and obesity as the 95th percentile or above (Sahoo et al., 2015). Based on criteria recommended by the World Health Organization, obesity is defining according to BMI for specific age groups, with a Z-score of >2 defined as overweight and that of >3 defined as obesity. In a multicentric study, children with a BMI of >30 kg/m2 were classified as obese and those with a BMI of > 25kg/m2 were classified as overweight. However, waist circumference was not measured as an indicator of overweight and obesity in children (aged 3–14 years) and adolescents (aged 15–18 years). The prevalence of obesity is considered to be a result of the interaction between genetic, psychological, cultural, socioeconomic, and environmental factors. In previous studies, sleep duration was assessed using self-reported or objective measurements (e.g. actigraph or polysomnography) (Reutrakul et al., 2018).

According to the National Growth Study, the occurrence rate of obesity was 11.3% in school-going children aged 5-18 years. Further, it was 11% in female and 7.8% in male students aged 5-12 years, and 13.8% and 12.1%, among students aged 13-18 years, respectively. Therefore, females were found to be more prone to obesity as compared to males (Al Dhaifallah et al., 2015).

Obesity in school children: Female students aged 5–18 years are more prone to weight gain and obesity as compared to their male counterparts owing to inherent hormonal differences. Consumption of fatty food, inconsistent exercise habits, and inactive lifestyle are the major reasons for obesity; however, apart from these factors, age, gender, family history, and parental lifestyle also lead to obesity (Birch and Fisher, 1998). Lee et al (Lee et al., 2000) confirmed that obesity is linked with socio-economic status, which is further connected with salary, occupation, and educational accomplishment. Among environmental factors, viewing television while consuming food was found to lead to obesogenic eating behaviour and further gain in body weight (Boswell et al., 2019). Polycystic ovarian syndrome (PCOS) was another factor linked with weight gain in female school-going children aged 12–16 years. The relationship between PCOS and obesity is linked to the high production of insulin, leading to irregular and impaired blood glucose level management (Bremer and disorders, 2010, Koivuaho et al., 2019, Witchel et al., 2019).

Apart from this, having a playground in school has also been found to play a critical role in weight management, specifically in female children. Katare et al 2019) confirmed that BMI is strongly influenced by environmental factors. Another previous study indicated that the consumption of sugary beverages; snacks with a high salt content; lower activity level; family history; and psychological factors such as anxiety, depression, dissatisfaction with one's body, eating disorders, and emotions lead to childhood obesity, specifically in school-going girls (Sahoo et al., 2015). Excess usage of electronic devices such as watching television for several hours and sedentary lifestyle have been found to contribute to obesity (Tuohino et al., 2019). However, until now, there is no clear strategy for the treatment of obesity [Table 1] (Cuda and Censani, 2018). It is important to note that clinicians and nutritionists can convert obesity from an irreversible to a reversible disease by developing and implementing appropriate guidelines.

Association between SSD and childhood obesity: Globally, a limited number of systematic reviews and meta-analyses have explored the link between SSD and childhood obesity. According to Li et al. (Li et al., 2017b), though several epidemiological and meta-analysis studies have been conducted to examine this relationship, most studies were based on cross-sectional observations. Patel et., al 2006 cconfirmed the strong association in women because participants whose sleep duration was between < 5–7 hours/day had gained a significant amount of weight. Cappuccio et., al 2008) conducted a meta-analysis on a global population of children (n = 30,002) and adults (n = 6,04,509) with SSD. They reported

a consistent increased risk of obesity among children and adults with SSD.

However, in their study on infants, Alamian et al 2016 reported a negative association between sleep problems and childhood obesity. In their meta-analysis on SSD and childhood obesity, Chen et al 2008) reported a significant inverse association between these two variables. Hart et al 2011) also confirmed that SSD is associated with weight gain, which in turn tends to lead to obesity. Matricciani et al 2012) examined data collected on 0.7 million children over the past 100 years, from 20 different countries. They confirmed that children's sleep duration was a minimum of 20–25 minutes lower than that of their parents when the latter were of their age. At night, the average sleep duration of school-age and preschool children was 9.4 and 9.6 hours/day, respectively, and that of toddlers and infants was 9.8 and 9.0 hours/day, respectively (2004., 2004). Ash and Taveras, (2017) have confirmed that insufficient sleep is associated with numerous adverse health outcomes in children.

Table 1. Diagnosing the BMI based on age factor (Reference: (Cuda and Censani, 2018))

Infancy (0-24 Months)	Toddler (2-4 Years)	Early childhood (5-9 Years)	Puberty (10-14 Years)	Adolescent (15-18 Years)	Adult (≥19 Years)
Weight > Length	BMI ≥ 95th Percentile or ≥85th percentile with couple or more risk factors	BMI ≥ 95th Percentile or ≥85th percentile with couple or more risk factors	BMI ≥ 95th Percentile or ≥85th percentile with couple or more risk factors	BMI ≥ 95th Percentile or ≥85th percentile with couple or more risk factors	BMI ≥ 95th Percentile or ≥85th percentile with couple or more risk factors
	• Fasting Blood Glucose/ HgA1c • Fasting Lipid Profile /Random Blood Sugar • ALT, AST and GGT • Consider 25 OH Vitamin D	• Consider sleep study • Consider liver ultrasound • Consider uric acid •Consider fasting serum insulin	• Consider urine microalbumin Ratio • Consider C-Peptide • Hs-CPR	• Consider urine microalbumin Ratio • Consider C-Peptide • Hs-CPR	

Miller et als (Miller et al., 2018) systemic review and meta-analysis confirmed that SSD is a risk factor for the development of obesity in infants, children, and adolescents. These findings were confirmed and corroborated by similar prior studies (Li et al., 2017b, Marin-Oto et al., 2019, Spinelli et al., 2019, Taveras et al., 2008, Wu et al., 2017, Zhang et al., 2019).

Recommendation: This review article recommends that school-aged children (aged 5–17 years) should receive a minimum of 9–12 hours of sleep per day to avoid future health complications, mainly weight gain, which may in turn lead to childhood obesity.

CONCLUSION

Sleep deprivation is associated with multiple hormonal responses which lead to appetite dysregulation, and negative effects on hunger and satiety, which in turn lead to low leptin and high ghrelin levels. The present review identified sleep as an important modifiable risk factor for obesity in infants, children, adolescents, and adults. Based on numerous studies, SSD is confirmed as a risk factor for obesity in infants, and children. Therefore, this review recommends the prevention of SSD to avoid future health complications.

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Dentistry Amid Corona Viral Disease-19: The Why, What, When and How of it ?

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ABSTRACT

Coronavirus disease (*COVID-19*) is a highly infectious disease which came to limelight in the beginning of the year 2020. With its rapid increase and spread, finally on 11th March, 2020, WHO declared the COVID-19 a pandemic disease . The possible routes of 2019- nCoV transmission are direct contact, droplet and aerosol transmission, fomite spread and feco-oral routes. Many dental procedures like tooth preparation, ultrasonic scaling, caries removal, etc produce aerosols and droplets which are contaminated with bacteria, viruses, and blood. These have the potential to spread infections to dental personnel and other people in the dental office. Not only this but also due to the characteristics of dental settings like closed air-conditioned space, the risk of cross infection may be high between dental practitioners and patients. A better understanding of aerosol transmission and its implication in dentistry can help us identify and rectify negligence in daily dental practice. It is crucial for dentists to refine preventive strategies to avoid the *COVID-19* infection by focusing on patient selection, hand hygiene, all personal protective equipment (PPE), and caution in performing aerosol-generating procedures. These special precautions elaborated in this paper would help control the spread of *COVID-19*.

KEY WORDS: COVID-19, CORONA, DENTAL PRACTICE.

INTRODUCTION

Coronavirus disease (*COVID-19*) is a highly infectious disease which came to limelight in the beginning of the year 2020. It spreads due to the newly discovered coronavirus which was first isolated on 7th January 2020 in Wuhan, China and was tentatively named as *2019-nCoV* on 17th January 2020 (Mukhtar and Mukhtar, 2020). The International Committee on Taxonomy of Viruses named the virus *SARSCoV-2* due to its resemblance to *SARS coronavirus* (Huffman, 2020). On 30th January 2020, the WHO declared this Chinese outbreak of *COVID-19* to be a Public Health Emergency of International Concern (PHEIC) as per the International Health Regulations (IHR, 2005) posing a high risk to countries with vulnerable health systems. On February 11, 2020, the WHO Director-General, Dr. Tedros Adhanom Ghebreyesus, named this disease "*COVID-19*," which is the acronym of "coronavirus disease 2019" (Cascella et al., 2020).

There was an unprecedented spread of the disease to 18 countries with four countries reporting human-to-human transmission. On February 28, 2020, WHO raised the threat to the *CoV* epidemic to "very high" level. Finally on March 11, as the number of *COVID-19* cases outside China had increased 13 times and the number of countries involved had tripled with more than 118,000 cases in

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114 countries and over 4,000 deaths, WHO declared the *COVID-19* a pandemic. In India it started to spread from early March, where only 3 cases were reported till 3rd March. Soon there was a spurt, and by 5th March, 29 cases had been reported; mostly in Delhi, Jaipur and Agra in Italian tourists and their contacts (Singhal, 2020). As of 1st August,2020 in India, there are 17,51,919 confirmed cases out of which 16,96,558 have recovered, while 37,403 are dead. Currently, worldwide 1,80,13,101 cases have been confirmed to be corona positive, out of which 1,13,26,866 have recovered while 6,88,289 are deceased (WHO, 2020).

The possible routes of *2019- nCoV* transmission are mainly direct contact and droplet transmission as stated in the 6th Edition of *COVID-19* Treatment Regimen (Trial Implementation) published by the National Health Commission of the People's Republic of China (2020). Aerosol transmission is also possible when there is an exposure to high concentrations of aerosols in a relatively closed environment (Ge et al., 2020). It is also critical to remember that the virus can survive on hands, objects or surfaces that were exposed to infected saliva in the previous nine days (Meng, Hua and Bian, 2020; Spagnuolo et al., 2020).

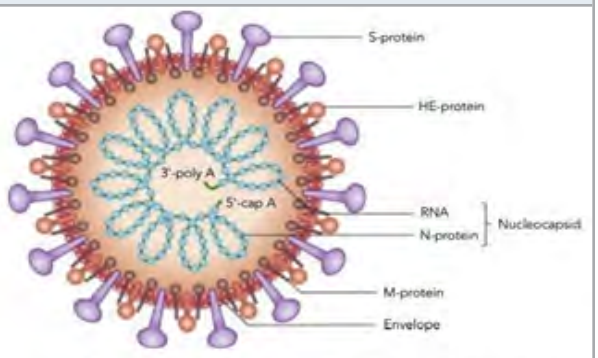
The evidence shows that many dental procedures like tooth preparation, ultrasonic scaling, caries removal, etc produce aerosols and droplets which are contaminated with bacteria, viruses, and blood. These have the potential to spread infections to dental personnel and other people in the dental office (Harrel and Molinari, 2004);(Ge et al., 2020). Not only this but also due to the characteristics of dental settings like closed air conditioned space, the risk of cross infection may be high between dental practitioners and patients. This leads us to understand the importance of strict and effective infection control protocols for dental practices and hospitals in countries/ regions that are (potentially) affected with *COVID-19*. It is crucial for dentists to refine preventive strategies to avoid the *COVID-19* infection by focusing on patient selection, hand hygiene, all personal protective equipment (PPE), and caution in performing aerosol-generating procedures (Meng, Hua and Bian, 2020). This paper aims to review and bring forth the necessary guidelines to be followed by dentists for their practice amid *COVID-19*.

The Corona Virus

Family: Coronaviruses belong to the family of *Coronaviridae* and order *Nidovirales*. Their genome consists of large, single, plus-stranded RNA (Gorbalenya et al., 2006; Fehr and Perlman, 2015). Currently, there are four genera of coronaviruses: α*CoV,* β-*CoV,* γ-*CoV, and* δ-*CoV* (Peng et al., no date), amongst which only the α-*CoV* and β-*CoV* mainly infect the respiratory, gastrointestinal, and central nervous system of humans and mammals (Perlman and Netland, 2009). This is not the first time the corona virus has spread throughout the world. Earlier *SARS-CoV* and the Middle East respiratory syndrome coronavirus (*MERS-CoV*) explored in 2002–2003 and in 2012, respectively, causing fatal severe respiratory diseases (Falsey and Walsh, 2003; Holmes, 2003). *2019-nCoV* which was explored in Wuhan, like *SARS-CoV* belongs to the β-*CoV* genera according to the phylogenetic analysis based on the viral genome and is considered to be more fatal and dangerous than the previous two types (Wu et al., 2020).

Structure:

Figure 1: COVID-19 (after WHO)



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A typical corona virus has a round or elliptic and often pleomorphic form, and a diameter of approximately 60–140 nm with 'spiked' protein in its membrane envelope (Li, 2016). It expresses other proteins like RNA polymerase, 3-chymotrypsin-like protease, papain-like protease, helicase, glycoprotein, and accessory proteins (Zhou et al., 2020). The S protein from coronavirus can bind to the receptors of the host to facilitate viral entry into target cells like the human angiotensin converting enzyme (ACE2) (Belouzard et al., 2012). *2019-nCoV* can effectively use ACE2 as a receptor to invade cells, which may promote human-to-human transmission. Like other *CoVs*, it is sensitive to ultraviolet rays and heat and can be effectively inactivated by lipid solvents including ether (75%), ethanol, chlorine-containing disinfectant, peroxyacetic acid and chloroform except for chlorhexidine (Cascella et al., 2020).

Manifestation: Patients with *COVID-19* usually present with clinical symptoms of dry cough and shortness of breath. In addition symptoms such as fever, chills, muscle pain, headache, sore throat, reduced sense of smell (hyposmia), and abnormal taste sensation (dysgeusia) have also been reported, (Guan et al., 2020). In addition, abnormal chest X-ray and computed tomographic findings such as ground-glass opacities are typically found in the chest. Notably, about 80% of these patients have only mild symptoms that resemble flu like symptoms and seasonal allergies, which might lead to an increased number of undiagnosed cases(Ather et al., 2020) Currently, in the world out of the infected cases, 21,88,010 (98%) cases have shown mild symptoms while 49,074 are in serious condition(2%)(WHO).

The incubation period of this disease can range from 0 to 24 days which allows its transmission to occur even before any symptoms pop up(Guan et al., 2020; Rothe et

al., 2020). The higher-risk patient population manifests symptoms typical of pneumonia or acute respiratory distress syndrome. Severe forms of this disease have a predilection for men with a mean age of 56 years with preexisting chronic illnesses such as cardiovascular disease or immunosuppression, (Ather et al., 2020). Some unconfirmed data suggest that Asian males have a large number of ACE2-expressing cells in the lung, which may partially explain the male predominance of *COVID-19*(del Rio and Malani, 2020).

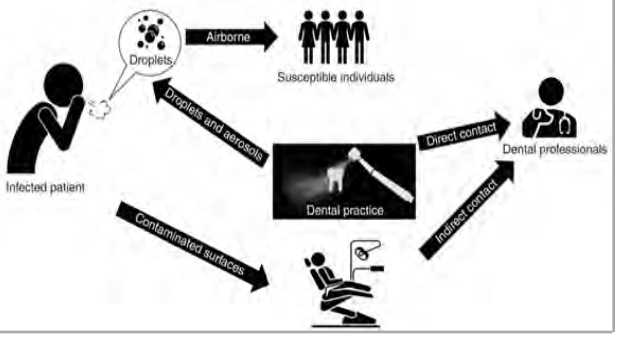
Modes of transmission: In general, any respiratory virus infection can occur through (Moriyama, Hugentobler and Iwasaki, 2020):Contact (direct or indirect), Droplet spray in short range transmission, Aerosol in long-range transmission (airborne transmission). The common transmission routes of novel coronavirus include direct transmission (cough, sneeze, and droplet inhalation transmission) and contact transmission (contact with oral, nasal, and eye mucous membranes)(Lu, Liu and Jia, 2020). The virus is thought to spread mainly between people who are in close contact with one another (about 6 feet) and through respiratory droplets produced when an infected person coughs or sneezes(centre for disease control). This had led to recommendations of social distancing.

Another important route of transmission is if droplets of *CoV-19* land on inanimate objects located nearby an infected individual and are subsequently touched by other individuals(for Disease Control and Others, 2020). In addition, studies have shown the presence of *SARS-CoV-2* in both saliva and feces of the affected patients (To et al., 2020; Zhang, Wang and Xue, 2020). Some people without symptoms may also be able to spread the virus (Chan et al., 2020) and transmission may occur before the disease symptoms appear.

Corona and dentistry: *SARS-CoV-2* can bind to human angiotensin converting enzyme 2 (ACE-2) positive cells (Zhou et al., 2020). The ACE2 are highly expressed on the mucosa of the oral cavity, epithelial cells of the tongue and also in the salivary glands. These findings have concluded that the oral cavity is a potentially high risk for *2019-nCoV* infectious susceptibility, (Xu et al., 2020) and also a possible explanation for the presence of *SARS-CoV-2* in secretory saliva (Hoffmann et al., 2020; Sabino-Silva, Jardim and Siqueira, 2020). Dental care settings involve face-to-face communication with patients, frequent exposure to saliva, blood, and other body fluids, and the handling of sharp instruments. This leads dental professionals to be at high risk for nosocomial infection, become potential carriers of the disease and also expose patients to cross-contamination (Fig 2).

Characteristics of dental set up which make it prone to spread of *CoV-19*: Production of bioaerosols : When performing dental procedures with a high speed handpiece or scaling using the ultra - sonic scaler, aerosols are generated(Organization and Others, 2020). These aerosols when combined with bodily fluids in the oral cavity, such as blood and saliva, create bioaerosols which are commonly contaminated with bacteria, fungi, and viruses and have the potential to float in the air for a considerable amount of time and be inhaled by the dentist or other patients (Grenier, 1995; Jones and Brosseau, 2015) leading to spread of disease. Based on the current epidemiological data, *2019-nCoV* has higher transmissibility than *SARS-CoV* and *MERS-CoV* (Chen, 2020) making dental clinics very prone to spread of infection.

Figure 2: Transmission of infection in dental setting.



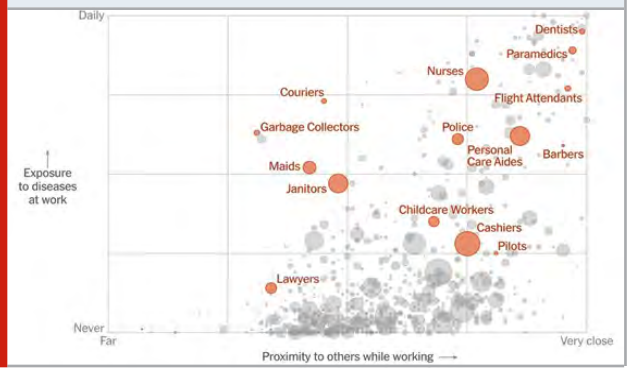
Small confined place: A dental clinic is a small confined place with an air conditioner whose strong airflow directs and propagates the droplet transmission. Most dental settings neither have airborne infection isolation rooms nor a respiratory protection program. Most of the equipment found in the dental settings do not withstand chemical sterilization and fumigation. Viruses can remain viable in aerosols for up to 3 hours and this makes dental clinic prone to spread of infection (van Doremalen et al., 2020).

Fomite spread: Human coronaviruses such as *SARS-CoV*, Middle East Respiratory *Syndrome coronavirus* (*MERS-CoV*), or endemic human coronaviruses (HCoV) can persist on surfaces like metal, glass, or plastic for up to a couple of days (Otter et al., 2016; Kampf et al., 2020) which make these contaminated surfaces in healthcare settings a potential source of coronavirus transmission. In dental practices, droplets and aerosols from infected patients are likely to contaminate the whole surface in dental offices. At room temperature, *CoV-19* remains infectious from 2 h up to 9 days, and persists better at 50% compared with 30% relative humidity. Thus, keeping a clean and dry environment in the dental office would help decrease the persistence of *2019- nCoV* (Peng et al., 2020).

Floor: In a study in China, the floor swab samples tested positive for *CoV-19* (ICU 70% , GW 15.4%), perhaps because of gravity and air flow causing most virus droplets to float to the ground(Guo et al., 2020). This brings to light that the floor itself can be contaminated in a dental setting and needs to be disinfected after every patient. In a news article by Lazaro Gamio, The New York Times, 15th March, 2020, the dental profession was shown to be at the highest risk amongst the health care workers (Fig 3). There is a potential for transmission

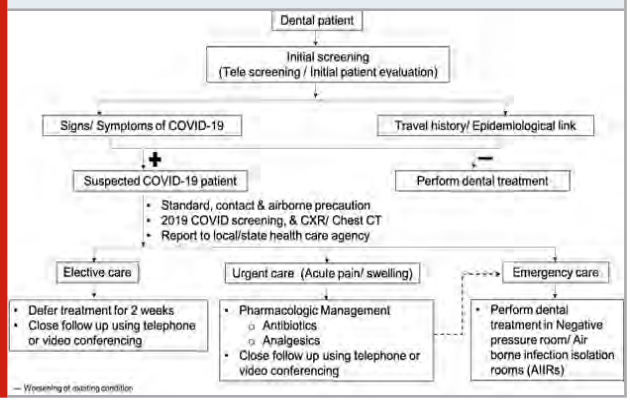
of *COVID-19* via aerosol, fomites or fecal-oral route that may contribute to nosocomial spread in the dental office setting (Peng et al., 2020). Therefore, modification of standard precaution and infection control regimen targeted toward *2019- nCoV* is essential during this outbreak.

Figure 3: Workers who face greatest coronavirus risk. (A news article in New York Times dated 15th March, 2020)



Our role as Dental Professionals (Jamal et al., 2020):a) Believe and follow reliable information.b) Avoid panic and rumors c) Take the recommendations from the local, state and government public health officials. d) Heed the call to temporarily suspend all non-urgent dental treatment until this crisis is over. Based on the experience gained from the previous outbreaks of *SARS-CoV*, data available on *SARS-CoV-2* and its associated disease (*COVID-19*) and recommendations provided from dental council of india and indian dental association, certain specific measures are discussed here for dental patient management in this epidemic period of *COVID-19* (Fig 4). On March 17, 2020, the Dental Council of India recommended that dentists postpone elective procedures for the country lockdown period and instead only provide treatment for dental emergencies.

Figure 4: Patient screening for COVID19 management.



Dental emergencies: American Dental Association says that dental emergencies are potentially life threatening and require immediate treatment to stop ongoing tissue bleeding, alleviate severe pain or infection, and include uncontrolled bleeding, cellulitis or any swelling that potentially compromise the patient's airway and trauma involving facial bones. Urgent dental care focuses on the management of conditions that require immediate attention to relieve severe pain and/or risk of infection. These should be treated as minimally invasively as possible and are as follows: Severe dental pain from pulpal inflammation pericoronitis or third-molar pain, surgical post-operative osteitis, dry socket dressing changes, abscess, or localized bacterial infection resulting in localized pain and swelling. Tooth fracture resulting in pain or causing soft tissue trauma, Dental trauma with avulsion/luxation, Dental treatment required prior to critical medical procedures, Final crown/bridge cementation if the temporary restoration is lost, broken or causing gingival irritation, Biopsy of abnormal tissue, Amid the covid scenario, treatment should be provided only if it falls in any of these categories.

Dentists who should avoid practice (Jamal et al., 2020):Above the age of 55 years.Having underlying systemic conditions like diabetes mellitus, chronic liver disease, heart and kidney diseases, lung conditions, cancer, pregnancy, foreign travel history in the last 28 days. Anyone suffering from cough/ cold/ fever

Precautions to be taken while practising dentistry:(A) Changes in Dental office: Waiting area management: Seating to be rearranged for social distancing. A spatial separation of at least 1m should be maintained between patients (Ge et al., 2020). Use of audio-visuals - These are posters or videos on TV screens regarding the behavioural changes like hand hygiene, respiratory hygiene, and cough etiquette to be followed during pandemic times (Fig 5).

Figure 5: Visual alert for sticking in the dental clinic.

Sign at entrance - Instructing patients having symptoms of a respiratory infection (e.g., cough, sore throat, fever, sneezing, or shortness of breath) to please reschedule their dental appointment and call their physician. Hand sanitizer upon entry and exit.Adequate ventilation required-For rooms with natural ventilation, 60 L/s per patient is considered adequate ventilation (World Health Organization, Chartier and Pessoa-Silva, 2009). Sensor devices like automatic hand sanitizer dispenser, auto liquid soaps, automatic door remote chair operating etc. can be introduced.

Patient Instructions: Patients should be informed that emergency work is given priority and elective procedures especially oral prophylaxis not recommended till further notice. Self declaration form to be filled by all the patients. This resolves legal as well as social stigma issues.Patients should visit the clinic only by appointment. Patient plus one attending person maximum that too if required. Face mask to be worn by patients as well as the accompanying person. Hand hygiene and respiratory instructions to be followed on how to use tissues to cover nose and mouth when coughing or sneezing, to dispose off tissues and contaminated items in waste receptacles immediately. Patients should cooperate and not put forth unreasonable demands.

Dental staff instructions: Front desk staff should be separated from the waiting room using transparent glass or barriers. No handshakes with the patients. Proper training to be given regarding the use and disposal of dental waste and PPE. Face mask to worn all the time.

Operatory management: The clinical team should wear gloves, mask, head cap and shoe covers at all times. Patients can remove mask at the time of oral examination and procedure (Attendee to wear mask full time). Multichair clinics without cabins should treat single patient at a time in case of aerosol procedure. Chair as well as the other contact surfaces like arm rest, light handle, tray handle etc should be wrapped in barrier film or disinfected after every patient. The barrier film should be changed after every patient. Air purifiers/ Filters to remove/filter contaminated air in treatment areas. Eg.High efficiency particulate arrestor (HEPA) filter, High volume evacuator(HVE).

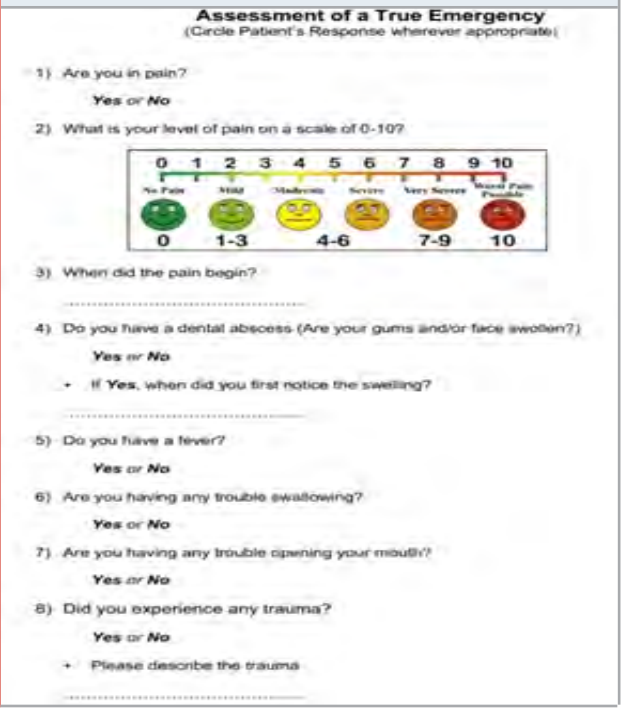
HVE filter - It is a suction device which helps to remove air at a rate of up to 2.83 m3 per minute and can effectively reduce contamination caused by the operating site by 90% (Narayana et al., 2016). However, it needs to be held at a proper distance (approximately 6–15 mm) from the active ultrasonic tip and always requires a dental assistant.

HEPA filter: It is an air filtration device that can remove 99.97% of the particles measuring 0.3 µm in diameter but it may become a source of microbes if the retained microorganisms proliferate and enter back into the filtered air(Chuaybamroong et al., 2010).

Negative pressure treatment room/Airborne infection isolation rooms (AIIRs): Patients with suspected or confirmed *COVID-19* infection should not be treated in a routine dental practice setting. Anticipatory knowledge of health care centers with provision for AIIRs would help dentists to provide emergency dental care if the need arises (Lai et al., 2020). Keep the AC vent facing upwards.

Precautions before treating patients: Patient evaluation and Cohorting: A detailed medical history form, *COVID-19* screening questionnaire and assessment of a true emergency questionnaire (Fig 7) to be filled by the patient.Measurement of patient's body temperature using a non-contact forehead thermometer or with cameras having infrared thermal sensors (Peng et al., 2020). Decide if treatment is required or not. Prescribe medications if not treating.

Figure 6: Assessment of true emergency form


Assessment of a True Emergency
(Circle Patient's Response wherever appropriate)

1) Are you in pain?
Yes or No
2) What is your level of pain on a scale of 0-10?
0 1 2 3 4 5 6 7 8 9 10
0 1-3 4-6 7-9 10
3) When did the pain begin?
4) Do you have a dental abscess (Are your gums and/or face swollen?)
Yes or No
- If Yes, when did you first notice the swelling?
5) Do you have a fever?
Yes or No
6) Are you having any trouble swallowing?
Yes or No
7) Are you having any trouble opening your mouth?
Yes or No
8) Did you experience any trauma?
Yes or No
- Please describe the trauma

Hand hygiene: Wash hands with soap and water for at least 20 seconds or use an alcohol-based hand sanitizer with at least 60% alcohol (Fig 8). Wash hands before and after any direct patient contact and between patients, whether or not gloves are worn, immediately after gloves are removed, before handling an invasive device, after touching blood, body fluids, secretions, excretions, non-intact skin, and contaminated items, even if gloves are worn, during patient care, when moving from a contaminated to a clean body site of the patient, after contact with inanimate objects in the immediate vicinity of the patient.

Personal Protective Equipment (See Fig 9): Hazmat Suit - required for aerosol procedure else surgical gown or surgical apron may suffice.Head caps- surgical

head caps or bouffant caps to protect hair. Disposables are preferable. Face Masks-Surgical Masks 3 or 4 ply adequate for regular checkup but N95 compulsory for aerosol procedure. Eye wear -sealed eyewear preferable like swimming goggles. In case of aerosol procedures, face shields are needed. Shoe covers - please avoid being barefoot both for doctor and patient. Shoe covers to be provided for all entering the clinic.Gloves - one pair of gloves at all times to prevent unnecessary touching of face and mouth and eyes etc., second pair during procedure. Preferably nitrile or surgical gloves when doing procedures. Proper sequence to be followed for wearing and removing the PPE.

Figure 7: Hand hygiene instructions.

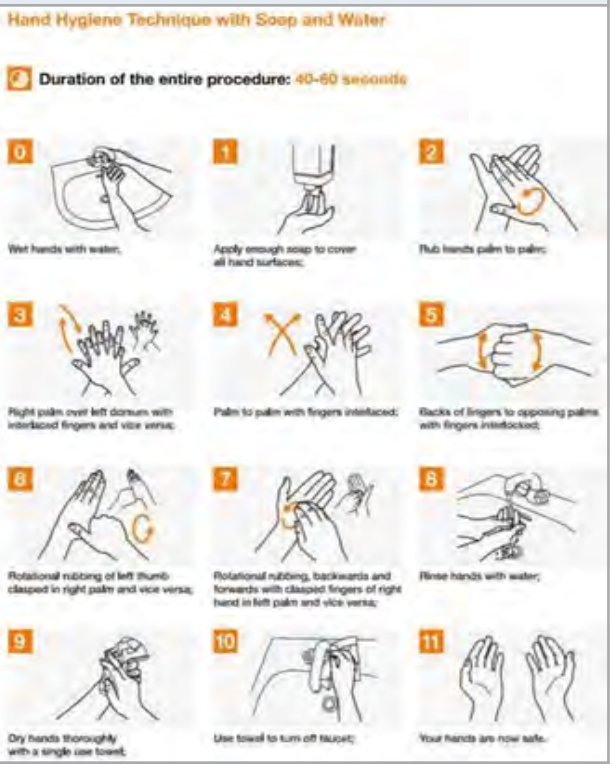
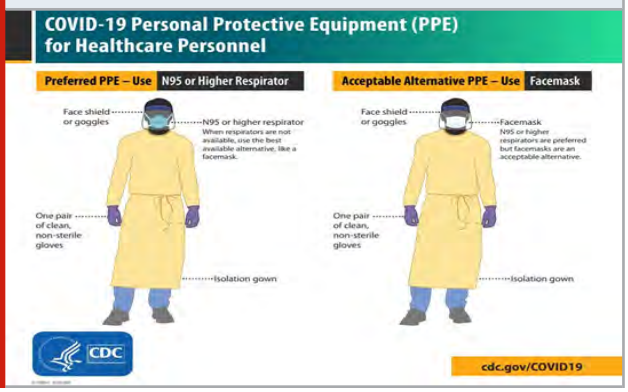


Figure 8: Personal protective equipment



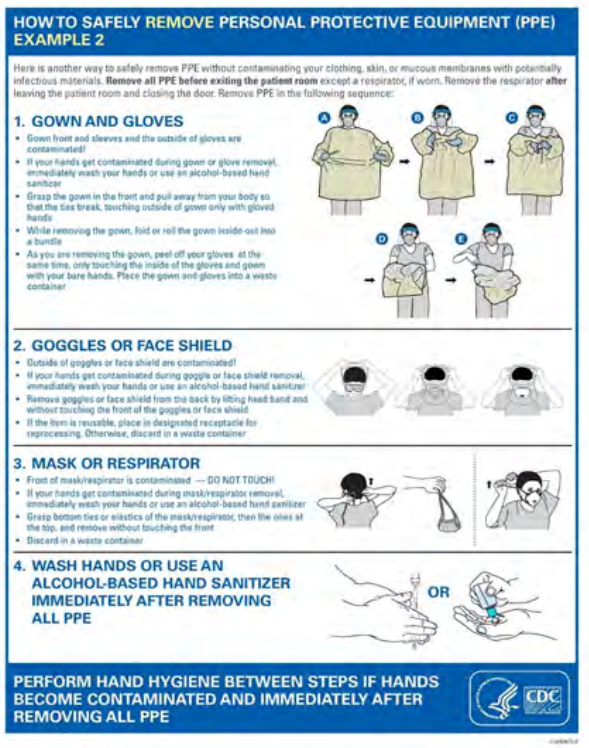
(C) Precautions while treating patients: Preprocedural mouth rinse: Patient to gargle with 0.2 % povidone iodine or 0.12% CHX for a full minute to reduce microbial load in saliva (Kariwa, Fujii and Takashima, 2006; Peng et al., 2020). Previous studies have shown that *SARS* and MERS were highly susceptible to povidone mouth rinse (Eggers et al., 2018).

Disposable devices: Use of disposable (single use) devices such as mouth mirror, syringes and blood pressure cuff to prevent cross-contamination.

Radiographs: Extraoral imaging such as panoramic radiograph or CBCT should be used to avoid the gag reflex or cough that may occur with intraoral imaging. When intraoral imaging is mandated, sensors should be double- barrier to prevent perforation and cross-contamination (Hokett et al., 2000).

Preferably no aerosol procedure: If required, then use: Continuous high volume suction to reduce aerosol spread. Rubber dam isolation to minimize splatter generation as it helps to shield the airway out reducing the chances of viral load in aerosols. It has been reported that the use of rubber dam could significantly reduce airborne particles in the 3-foot diameter of the operational field by 70%(Samaranayake, Reid and Evans, 1989). Anti-retraction hand pieces with specially designed anti-retraction valves or other anti-reflux designs are strongly recommended as an extra preventive measure for cross-infection(Samaranayake and Peiris, 2004). They significantly reduce the backflow of oral bacteria and virus into the tubes of the hand piece and dental unit as compared with the hand piece without anti-retraction function(Hu et al., 2007).

Figure 9: Removal sequence of PPE.



Precautions after treating patients:
Disposal of PPE: One should follow the correct removal sequence of PPE (Fig 10).It is recommended to dispose off each set of PPE in individual bags.

Management of medical waste: The reusable instrument and items should be pretreated, cleaned, sterilized, and properly stored.The medical waste should be transported to the temporary storage area.Double-layer yellow color medical waste package bags and "gooseneck" ligation should be used for medical and domestic waste generated by the treatment of patients with suspected or confirmed *2019-nCoV* infection as they are regarded as infectious medical waste, (Peng et al., 2020).

Disinfection of clinical settings: Separate shoe covers for everyone entering the operatory to prevent spread through the floor. Surface disinfection with aldehyde free solutions is recommended eg, Bacillol, Surfasept etc.Floor cleaning should be done by wet mopping with surgical floor cleaners or hypochlorite solution, use of broom is not advised. All these precautions should be followed once the dentist decides to start his/her clinical practise.

Prosthodontists to Special care for few procedures (Ge ZT et al):

1. Impressions to be washed with water and disinfected with cold sterilisation like cidex or sprays like Dimenol.
2. Extreme care to be taken while transfering materials to and from the dental lab.
3. Gagging should not occur while suctioning.
4. Correct impression trays to be selected and adjusted for avoiding cough reflex. For highly sensitive patients, consider applying oral mucosa anesthesia to the throat before impression taking.
5. During tooth preparation try to incorporate rubber dam application. For example, design supra-gingival margin for posterior bridge or use a split-dam technique (Li et al., 2004)
6. During removable partial denture or complete denture try-in, avoid touching other objects in the dental office after contacting patients' saliva.
7. Endodontic procedures under rubber dam only. Atraumatic restorative techniques instead of airotor for caries removal.
8. For oral prophylaxis, manual scaling and polishing is recommended over ultrasonic in required cases.

Reopening guidelines after lockdown (Centre for disease control and prevention): Develop your plan:Determine what needs to be cleaned (areas unoccupied for 7 or more days require only routine cleaning), how areas will be disinfected (prioritize disinfecting frequently touched surfaces) and consider resources and equipment needed (availability of cleaning products and personal protective equipment (PPE) appropriate for cleaners).

Implement: Clean visibly dirty areas with soap and water, use appropriate disinfectant product (EPA- approved disinfectant against *COVID-19*) and always follow the safety information and application instructions.

Maintain and Revise: Continue routine cleaning and disinfection (disinfect frequently touched surfaces at least daily), maintain safe practices (frequent hand washing, face masks and staying home if sick, maintaining social distancing)

CONCLUSION

Dentistry being highly prone to spread of infection, we as dental professionals should take utmost care and precautions while practicing it. In India, the dental council of India released a circular explaining all the preventive and precautionary measures to be taken by the dentists amid *COVID-19*. It also announced that all dentists should be performing only emergency procedures in support of the country's lockdown initiated by the government of India until further notice. The emergence of *COVID-19* has brought new challenges and responsibilities to dental professionals.

A better understanding of aerosol transmission and its implication in dentistry can help us identify and rectify negligence in daily dental practice. In addition to the standard precautions, implementation of special precautions like patient evaluation, hand hygiene, personal protective measures, mouth rinse before dental procedures, rubber dam isolation, anti-retraction hand piece, disinfection of the clinic settings, and management of medical waste could prevent disease transmission from asymptomatic carriers. These special precautions would help control the spread of *COVID-19*.

Conflict of Interest: The authors declare no conflict of interest, financial or otherwise.

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The Effects of Salivary pH on Color Stability and Surface Roughness of Different Denture Acrylic Resin Materials

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ABSTRACT

A knowledge of color and surface roughness of denture base materials is important for achieving clinically successful complete dentures. The aim of this study was to evaluate the effects of different salivary pH values on color stability and surface roughness of heat cured, light cured and CAD/CAM fabricated denture acrylic resin materials. Thirty discs with dimensions of (10 × 3 mm) were fabricated from heat-cured, light-cured, and CAD/CAM denture acrylic resin materials. The color (ΔE) and surface roughness (Sa) were measured prior to the conduction of the experiment and after 30 days immersion in saliva using a Reflectance Spectrophotometer and non-contacting Profile-meter, respectively. All the acrylic resin specimens were subjected to brushing and thermocycling according to a standardized protocol. The discs of each type of acrylic resin materials were immersed and incubated in three different salivary pH values (acidic 5.7, neutral 7 and basic 8.3) for 30 days. Results were analyzed by two-way ANOVA followed by independent sample t-test for comparison. Both, the type of the acrylic resin material and the salivary pH value, have significant effects on color stability (ΔE) and surface roughness (Sa). Both heat cured and CAD/CAM fabricated acrylic resin materials exhibited clinically acceptable color values (ΔE<3.3) regardless of the salivary pH value utilized for incubation, this was not apply for light cure acrylic (ΔE>3.3) after being subjected to acidic saliva pH (ΔE = 7.29). CAD/CAM fabricated acrylic resin material exhibited the least amount of surface roughness following incubation in different salivary pH values. The study concluded that CAD/CAM fabricated denture acrylic resin material might be the material of choice to construct dentures for patients known to have acidic dietary intake. It demonstrated clinically acceptable color stability and lower surface roughness values in comparison to the other denture acrylic resin materials.

KEY WORDS: COLOR STABILITY, DENTURE ACRYLIC SALIVARY PH, SURFACE ROUGHNESS.

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INTRODUCTION

Complete dentures can be fabricated using different methods. The goal of each method is to produce a prosthesis that shows excellent mucosal adaptation resulting in good retention and stability (Bilgin et al., 2015). Polymethyl methacrylate (PMMA) resin has been successfully used for dentures base materials for many years, due to low cost, good physicochemical properties and acceptable esthetics. Moreover, it is easily processed, repaired and polished (Singh et al., 2013; Zuo et al., 2016). With the development of computer assisted design and computer assisted manufacturing (CAD/CAM) technology, complete dentures can be fabricated without the need for flasking or other processing methods. Additionally it has desired outcomes and great accuracy of fitting (Kattadiyil et al., 2015; Goodacre et al., 2016; Wimmer et al., 2016). Regardless of the fabrication technique used acrylic resin susceptible to many environmental factors, which may compromise its properties. These factors include temperature changes, humidity, and saliva. Salivary pH changes had been one of the main concerns in field of removable dental prosthesis (Muddugangadhar et al., 2015; Sonthalia et al., 2016; Tango et al., 2018).

The effects of the aforementioned factors on color stability of denture acrylic resin materials were well-established by Liberman et al., (1995) with color stability of dental prosthesis being a concern as it is related closely to aesthetics and patient satisfactions. The color of denture base materials should remain stable during clinical service and any changes in denture base materials color are an indicator of aging and material damage. The color stability of acrylic resin denture base and teeth by different beverages has been investigated (Mutlu-Sagesen et al., 2001; Imirzalioglu et al., 2010; Altinci and Durkaya, 2016; Alp G et al., 2019; Al-Qarni et al., 2020). The surface roughness (Sa) of denture base materials is affected by material properties, polishing techniques and the dental hygiene habits of patients. The Sa has an impact on patient comfort and esthetics and play a key factor in plaque accumulation that leads to denture stomatitis, staining and halitosis (Kuhar et al., 2005; Mörmann et al., 2013; Gungor H et al., 2014; Sahin et al., 2016; Darwish et al., 2016; Alp G et al., 2019).

Since complete dentures are often subjected to oral saliva with alternating states of alkalinity to acidity, studying the potential effects of the salivary pH on the properties of the acrylic resin is essential. Although the color stability and surface roughness of heat cured and light cured denture base materials have been reported studies of the color changes and surface roughness of CAD/CAM denture base materials are insufficient. The null hypothesis was that different salivary pH values have no effects on color stability, surface roughness of various types of denture acrylic resins materials.

MATERIALS AND METHODS

Three types of denture acrylic resin materials were utilized in this study; Heat cured (SR Ivocap High Impact®, IvoclarVivadentAG, Liechtenstein), Visible-light-activated resin (Eclipse®, Dentsply, United State), and CAD\CAM fabricated denture acrylic resin materials (IvoBase® CAD, Zenotec,Wieland Dental, Germany). Three groups of thirty discs each were fabricated with dimensions of 10 mm (diameter) × 3 mm (thickness). Each of the three groups was divided into three subgroups, with 10 discs each.

Acrylic Samples preparation: Fabrication of CAD\CAM denture acrylic resin discs: The discs were designed and milled using Zenotec® CAD software (Wieland Digital Denture Ivoclar Vivadent, Schaan, Liechtenstein) according to the predetermined dimensions. Poly methyl methacrylate (PMMA) blocks were used. The discs were then finished and polished using dental laboratory polishing machine with vacuum cleaner (ASPYCLEAN+ M2V®, Manfredi, Italy) with the use of Pumice (Pumice, INTERDENT, Slovenia) and rag polishing wheel (Rag Muslin wheel , Kerr, USA).

Fabrication of heat cured denture acrylic resin discs: A putty mold of the preferred disc dimensions was fabricated using putty polyvinyl siloxane material (Express STD®, 3M ESPE, United State). The silicone molds were filled by melted base plate wax (Figure 1). A Bantam flask was filled up with a plaster mix and then the putty mold was immersed. After that the flasks were placed in a wax elimination machine for 30 minutes at 90 -100 F0. The heat cured denture acrylic resin was then mixed for 5 minutes using a cap vibrator (Cap vibrator®, Ivoclar Vivadent, Schaan, Liechtenstein). The mixture was poured into the putty mold and pressed using pressure apparatus (OL 463, Manfredi, Italy). Then, the Flask assembly was placed in a polymerization path (100 C water) for 35 minutes (Electronic Denture Curing System, NevinLabs™, USA).The discs were finished and polished on the same manner.

Figure 1: The mold filled up with melted base plate wax.

Fabrication of visible-light-activated denture acrylic resin discs: The putty mold made of polyvinyl siloxane material (Express STD®, 3M ESPE, United State) was used to fabricate the light cured acrylic resin discs. According to the manufacturer's instructions, a thin layer of separator was painted on the mold's surface and

a small amount of visible light activated denture acrylic resin (Eclipse, dentsply, United State) was placed into the disc's mold. A thin layer of Eclipse Air Barrier (Eclipse Air Barrier Coating, DENTSPLY, USA) was applied to the top of the discs. The discs were then placed in the Eclipse Processing Unit for 10 minutes at +140 F◦, and then it was allowed to bench cool at room temperature. Then, the discs were polished on the same manner.

Measurement of color stability: Reflectance spectrophotometer (color-Eye 7000A®, Gretag Macbeth, NY, USA) was used for the color stability measurements of all the samples before and after incubating the discs in different pH values of artificial saliva. Color reading was performed using an 8-degree observer and illuminant D65. Color stability assessments were conducted in three randomly selected areas near the center of each acrylic sample. The average of the three readings was recorded and the mean color change of each sample was calculated using the CIE Lab uniform color scale. The level of the total color difference was formulated by a single number ΔE.

$$\Delta E=[(\Delta L^*)2+(\Delta a^*)2+(\Delta b^*)2]1/2\Delta E=(\Delta L^*)2+(\Delta a^*)2+(\Delta b^*)21/2$$

Where L* stands for lightness, a* for redness-greenness, and b* for yellowness–blueness (Asal et al., 2015).

Measurement of surface roughness: The surface roughness of the denture acrylic resin samples was determined with a non-contacting profile-meter (3D Opticalmicroscope contour GT-K1®, Bruker, United State). A 3D parameters of surface roughness with total of three readings were taken for each sample. The average of three readings of arithmetic mean height (Sa) was recorded in micrometers and the mean surface roughness change of each sample was obtained.

Na_2HPO_4	0.260g/l
NaCl	0.700g/l
KSCN	0.330g/l
KH_2PO_4	0.200g/l
$NaHCO_3$	1.500g/l
KCl	1.200g/l

Brushing protocol of the samples: The mechanical brushing test was performed following the recommendations of the International Organization for Standardization (2001). The specimens were brushed with soft tooth brushes mounted on a toothbrush simulator (toothbrush simulator ZM-3.12, SD Mechatromik GmbH,Germany) then the specimens were subjected to linear toothbrush abrasion movement with a rate of 356 brush strokes (back and forth) per minute. The machine provides a 200 g vertical load over each specimen and 5mm path starting from center of each specimen. The total brushing time was 50 minutes with total of 17800 cycles (representing one year). Brushing was carried out in distilled water (23+3 C0) and dentifrice (Crest Cavity Protection Regular Paste, P&G, Germany) (Hussein and Al-Ameer, 2012). Artificial saliva preparation and incubation of the samples: The artificial saliva was prepared in three different pH values (5.7, 7 and 8.3). An electrolyte composition similar to that of human saliva was used in this study (Pusz et al., 2010). This included the followings:

Buffer solution from KH_2PO_4 and Na_2HPO_4 was prepared by dissolving each one in 1 liter of de-ionized distilled water. Basic saliva was prepared by taking 500 ml of Na2HPO4 and adding KH_2PO_4 gradually to it until the exact pH was reached. Neutral and acidic saliva were prepared by slightly adding of Na_2HPO_4 to 500 ml of KH_2PO_4 until the exact pH was reached. For neutral saliva, greater amount of Na_2HPO_4 were added to reach the exact pH (Kostic et al., 2015).The discs were assorted into 9 groups, with 10 discs in each group, then stored in the artificial saliva in an incubator (blanket warming cabinet, MALMET, Australia) at 37oC for a total of 30 days.

Thermo-cycling protocol: Using SD Mechatronik GmbH thermo-cycler (SD Mechatronik, Germany), all specimens were stored in distilled water, going through thermo-cycling between 5 ^{0}C and 55 ^{0}C with a dwell time of 30 seconds, and a transfer time of 12 seconds for 1000 cycles (Oliveira et al., 2010).Data were analyzed using statistical software SPSS (v16, SPSS Inc., Chicago, IL, USA). The effect of acrylic material type and pH values and the interaction between them on color changes and surface roughness were analyzed using two-way ANOVA. Paired sample t-test was used to examine the difference between the pre and post color and surface roughness of all the acrylic material types.

RESULTS AND DISCUSSION

The Effects of pH Value on Post color stability on different denture acrylic resin materials: Two-way ANOVA was used to evaluate the effects of acrylic material type and salivary pH value on ΔE, and it was found that each independent factor had a significant effect on ΔE (Table 1).

The mean and standard deviation of each material when soaked in different salivary pH values are presented in (Table 2). When acrylic materials were soaked in acidic pH, the post ΔE of the CAD/CAM and Light Cure materials were higher than the pre ΔE (1.56 ± .56, 7.29 ± 3.14 respectively), while the pre ΔE of the Heat cure was higher than the post ΔE (3.07 ± 1.23).

When the different acrylic materials were soaked in saliva of neutral pH value, the pre ΔE of CAD/CAM and light cure materials were higher than post ΔE (1.35 ± 0.85, 4.28 ± 2.33), while the post ΔE of Heat Cure was higher than the pre ΔE (2.80 ± 1.43). The same pattern was observed when the materials were soaked in saliva of basic pH value (the pre ΔE of CAD/CAM and light cure materials were higher than post (1.86 ± 0.78, 4.28 ± 1.31 respectively), and the post ΔE of the Heat Cure material was higher than pre (2.62 ± 1.64). The independent

sample t-test revealed that those differences were found to be statistically significant only in the light cure material when soaked in saliva of acidic pH, and in the CAD/CAM and light cure materials when soaked in saliva of basic pH value ($p \leq 0.05$) as shown in (Table 3).

Table 1. Two way ANOVA of the effect of two independent variables material type and pH value on ΔE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Acrylic disc material	819.8	2	409.90	156.07	.000
PH	117.45	2	58.72	22.36	.000
Acrylic disc material * pH	120.50	4	30.13	11.47	.000
Error	1394.60	531	2.63		
Total	6840.60	540			
Corrected Total	2452.35	539			

a R Squared = .431 (Adjusted R Squared = .423)

Table 2. Difference of ΔE of different acrylic material after soaking in different pH values

pH	Acrylic disc material	ΔE	Mean	Std. Deviation
Acidic	CAD/CAM	Pre	1.49	.61
		Post	1.56	.56
	Heat Cure	Pre	3.07	1.23
		Post	2.94	1.39
	Light Cure	Pre	4.66	1.87
		Post	7.29	3.14
Neutral	CAD/CAM	Pre	1.35	.85
		Post	1.23	.58
	Heat Cure	Pre	2.59	1.29
		Post	2.80	1.43
	Light Cure	Pre	4.28	2.33
		Post	3.42	2.25
Basic	CAD/CAM	Pre	1.86	.78
		Post	.82	.93
	Heat Cure	Pre	2.57	.78
		Post	2.62	1.64
	Light Cure	Pre	4.28	1.31
		Post	2.47	1.62

Table 3. Independent Sample T-test between the pre and post ΔE of different acrylic materials under different salivary pH values.

pH	Acrylic Disc material	T-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
Acidic	CAD/CAM ΔE	-.48	58	.64	-.07
	Heat Cure ΔE	.40	58	.70	.144
	Light Cure ΔE	-3.94	58	.00*	-2.63
Neutal	CAD/CAM ΔE	.64	58	.53	.12
	Heat Cure ΔE	-.58	58	.56	-.20
	Light Cure ΔE	1.47	58	.15	.88
Basic	CAD/CAM ΔE	4.67	58	.00*	1.04
	Heat Cure ΔE	-.16	58	.89	-.05
	Light Cure ΔE	4.79	58	.00*	1.81

The effects of pH value on post surface roughness under different types of denture acrylic resin materials: Two-way ANOVA was used to evaluate the effect of acrylic material type and salivary pH value on Sa, and it was found that each independent factor had a significant effect on surface roughness (Table 4).

The mean and standard deviation of Sa of each material when soaked in different salivary pH values are presented in (Table 5). When acrylic materials were soaked in acidic pH, the pre Sa of the Heat cure and CAD/CAM materials were higher than the post Sa (1.59 ± .67, .18± .03 respectively), while the post Sa of the Light cure was higher than the pre Sa (.34±.24). The same pattern was observed when the materials were soaked in saliva of Neutral pH value, the pre Sa value of Heat cure and CAD/CAM materials were higher than post Sa (1.86±.57, .18±.03 respectively), and the post Sa value of the Light cure material was higher than pre Sa (.34± .15). When the different acrylic materials were soaked in saliva of Basic pH value, the pre Sa value of heat cure, light cure and CAD/CAM materials were higher than the post Sa (1.78±.63, .29±.08, .19±.03 respectively).

The independent sample t-test revealed that those differences were found to be statistically significant only in the CAD/CAM material when soaked in acidic, neutral and basic saliva, and in the Heat cure material when soaked in basic saliva ($p \leq 0.05$) (Table 6).

Based upon the results obtained from this study, the null hypothesis was rejected, meaning that the variation

in salivary pH values had a significant effect on color stability and surface roughness of the acrylic materials used in the study. Acrylic resin is one of the most popular and extensively used materials in different fields of medicine, especially in dentistry with prosthetic replacement and rehabilitation of missing structures being one of the main benefits gained from this material (Wiatrak et al., 2017). Despite the wide use of acrylic materials, many environmental factors are known to have adverse effects on its physical properties and clinical performance. Salivary pH fluctuation is considered as one of the main factors that might affect the performance of intraoral prosthesis, (Haug et al., 1999a; Haug et al., 1999b; Eleni et al., 2009; Sonthalia et al., 2016).

Table 4. Two way ANOVA of the effect of two independent variables material type and pH value on Sa values.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	233.67(a)	8	29.21	158.15	.00
Intercept	261.89	1	261.89	1418.02	.00
Disc material	230.32	2	115.16	623.55	.00
pH	1.25	2	.63	3.39	.04
Disc material * pH	2.1	4	.52	2.84	.02
Error	98.07	53	.19		
Total	593.63	54			
Corrected Total	331.74	54			

a R Squared = .704 (Adjusted R Squared = .700)

Effects of Salivary pH Values Alteration on Color of Different denture Acrylic resin Materials: In the current study, color changes of CAD\CAM and heat cure acrylic materials were considered clinically acceptable (ΔE< 3.3) after subjected to 30 days of immersion in different salivary pH. This did not apply for light cure acrylic (ΔE > 3.3) after being subjected to acidic saliva pH (ΔE = 7.29). The effect of soaking the acrylic materials in acidic solutions- not necessary saliva- were investigated previously. Khan et al., (1987) compared the staining ability of light-cure acrylic and heat-cure acrylic resins when soaked in tea (acid). Their findings indicate that light-cure acrylic is 4 times more vulnerable to staining due to difference in water sorption between the two materials. Um and Ruyter, (1991) reported that coffee discoloration is due to both surface adsorption and absorption of colorants while with tea only adsorption of colorants was noticed. However, it is worth mentioning that these studies immersed the acrylic materials in colored solutions while in the current study clear artificial saliva was used. Similarly, our results were consistent with studies performed by Mutlu-Sagesen et al., (2001) and Koksal et al., (2008) who investigated the color stability of denture teeth when immersed in different solutions. Light cure acrylic material had the highest amount of color change when soaked in acidic saliva, which indicates that this material is least stable in the acidic environment.

Table 5. Difference of Surface roughness of different acrylic material after soaking in different pH values.

pH	Acrylic disc material	SR	Mean	Std. Deviation
Acidic	CAD/CAM	pre	.18	.03
		post	.15	.03
	Heat Cure	pre	1.59	.67
		post	1.51	.61
	LIGHT Cure	pre	.30	.14
		post	.34	.24
Neutral	CAD/CAM	pre	.18	.03
		post	.15	.02
	Heat Cure	pre	1.86	.57
		post	1.75	1.1
	Light Cure	pre	.29	.11
		post	.34	.15
Basic	CAD/CAM	pre	.19	.03
		post	.16	.03
	Heat Cure	pre	1.78	.63
		post	1.21	.59
	Light Cure	pre	.29	.08
		post	.26	.07

Table 6. Independent Sample T-test between the pre and post Surface roughness of different acrylic materials under different salivary pH values.

pH	Acrylic disc material	T-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
Acidic	CAD/CAM Sa	4.35	58	.00*	.03
	Heat Cure Sa	.44	58	.66	.07
	Light Cure Sa	-.63	58	.53	-.03
Neutral	CAD/CAM Sa	5.11	58	.00*	.04
	Heat Cure Sa	.48	58	.63	.11
	Light Cure Sa	-1.24	58	.22	-.04
Basic	CAD/CAM Sa	2.45	58	.02*	.02
	Heat Cure Sa	3.62	58	.00*	.57
	Light Cure Sa	1.14	58	.26	.02

* The mean difference is significant at the .05 level.

Imirzalioglu et al., (2010) evaluated the effect of different solutions of different pH values on the color stability of acrylic resin. It was concluded that acidic solution such as coffee and tea showed the highest amount of color

change. This was attributed to the unfavorable physical properties of the material where changes due to coffee (more acidic) were more than tea (weaker acid). On the contrary, another study that evaluated the effect of peracetic acid and sodium hypochlorite on acrylic resins, and found that there is no significant color changes irrespective of immersion time (Fernandes et al., 2013). However, this lack of difference was contributed to little immersion time/day and the short study period.

As stated before the color changes of CAD\CAM and heat cure acrylic materials were considered clinically acceptable ($\Delta E < 3.3$) this finding coincide with (Altinci and Durkaya, 2016; Alp et al., 2019; Dayan et al., 2019; Al Qarni et al., 2010). On the other hand this was not apply for light cure acrylic ($\Delta E > 3.3$) after being subjected to acidic saliva pH ($\Delta E = 7.29$) (Koksal et al., 2008; Bonatti et al., 2009; Canadas et al., 2010; Goiato et al., 2013; Fernandes et al., 2013). From the perspective of color stability, CAD\CAM acrylic materials showed high level of color stability that indicate higher clinical performance in terms of color stability and probably more patient satisfaction, on the other hand, light-cure acrylic showed high level of color deterioration that renders it to be the last choice when seeking high esthetic and color stability over time.

Effects of Salivary pH Values Alteration on Surface Roughness of Different denture Acrylic resin Materials: In the current study, it was found that the surface roughness of heat cure acrylic material significantly increased when soaked in neutral and basic pH values. This could be attributed to the fact that the pH values can affect the degradation rates of the polymers where the breaking strength of the polymer was found to depend markedly on the pH and was found to be highest at neutral pH (Achim, 1996; Hussein and Al-Ameer, 2012)). It was found that in basic pH values there is high number of Hydroxyl ions, which is responsible for accelerating the degradation, thus increasing the surface roughness (Cilli et al., 2012).

Our results demonstrated that the surfaces of the CAD/CAM and light cure acrylic materials were the least affected by changes in the pH values, meaning that both materials' surface roughness are resistant to environmental pH changes. According to Bagheri et al., (2007) the type of storage solution and the composition of the soaked material are important factors for the polymer degradation of the materials. Factors such as the solubility parameter, the cross-link nature of the resin matrix, and the solvent sorption uptake may influence more directly the polymer's degradation rate. Filler-Matrix de-bonding may occur in presence of water as diffusion of water occur at interface between filler particles and the matrix causing hydrolytic degradation and erosion of resin based material. Consequently, the combination of these factors may affect the wear undergone by the material (Ferracane, 2005; Cilli et al., 2012; Munchow et al., 2014). The ability of CAD/CAM to have smooth surfaces might be more material-related (Riccardo, 2016). Industrially fabricated CAD/CAM materials have a less risk of porosities and therefore higher mechanical properties (Stawarczyk et al., 2015).

The light cure acrylic material have the least favorable surface roughness in acidic and neutral pH values. This may be due to loss of structural ions that lead to softened of polymer surface (Gadelmawla et al., 2002). The low pH values can also change the urethane dimethacrylate resin matrix by acting as a catalyst for the ester groups that are present in dimethacrylate monomers. This process lead to a phenomenon known as plasticization formed due to degradation of the polymer network, that may increase the surface roughness of the resin (Ferracane, 2005; Miranda et al., 2011). Generally speaking, the CAD/CAM acrylic material demonstrated the best surface smoothness regardless of the salivary pH values it was soaked in. To the contrary, heat cure acrylic material demonstrated the worst surface smoothness regardless of the soaking salivary pH value. It is known that the salivary pH value continuously alters in the oral cavity between acidic and basic based on the dietary intake of the patient. Consequently, it might be necessary to subject the same acrylic material to altered salivary pH values and study its effect on surface roughness and color stability.

CONCLUSION

Within the limitations of the current study, it can be concluded that CAD/CAM acrylic materials are best used in patients with acidic dietary intake. CAD/CAM acrylic material exhibited clinically acceptable color stability and the least amount of surface roughness in comparison to the heat and light cure acrylic materials. Based on the results of this study, further studies should be performed to address the effect of different polishing techniques on the color stability and surface roughness of denture base materials.

Conflict of interest: The authors declare that they have no conflict of interests for the present study and all authors have read and approved the final draft.

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Indole Acetic Acid Production and its Quantification from Microorganisms of *Rhizosphere*

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ABSTRACT

Plant Growth Promoting Rhizobacteria (PGPR) in the category of microbes that reside in the roots of the plant as well as free in the soil have plant growth-promoting capability. Moreover, that has characteristic properties that are helpful for better productivity of plants mainly including, plant protection against pathogens, root and shoot elongation, and works as fertilizers. This study aims to identify and characterize PGPR isolates highlighting their Indole Acetic Acid production capability. *Pseudomonas aeruginosa* and *Bacillus cereus* were characterized by 16S rDNA sequencing and phylogenetic analysis was performed. PGPR characteristics were tested by Indole acetic acid,hydrogen cyanide, siderophore, and 1-aminocyclopropane-1-carboxylic aciddeaminase test. Quantification of the IAA production by the two isolates was done using Reversed phase High Performance Liquid Chromatography against standard indolic compounds. Both the isolates showed a comparable number and quantity of indolic compounds in their supernatant. The area under the authentic IAA peak was 97% of the total peak area, while the IAA peak extracted from the culture of strain LC536053 was 30% of the total peak area. Once calculated back to the original concentration of the culture extract based on comparison with the known authentic IAA concentration, it was found that the strain LC536054 produced approximately 118 µmol/mL of IAA.

KEY WORDS: PGPR, IAA, PLANT GROWTH, HPLC, UV DETECTOR, RHIZOSPHERIC, CHROMATOGRAM.

INTRODUCTION

PGPR's have an immense role in enhancing the growth of plants by producing various products that help to influence natural characteristic properties of plants. These bacteria can colonize in both the roots of the plants as well as rhizospheric soil, they also reside freely in the soil. The properties include the production of auxin, gibberellin, ethylene, siderophores, HCN, and antibiotics (Arshad and Frankenberger, 1992) which directly affects plant growth. They belong to varied genera *viz: Azospirillum, Alcaligenes, Arthrobacter, Enterobacter, Erwinia, Acinetobacter, Bacillus, Burkholderia, Flavobacterium, Pseudomonas, Rhizobium and Serratia* (Tilak et al., 2005). The Plant Growth Promoting Rhizobacteria (PGPR) are of the great importance than other plant growth promoting microorganisms because they are associated to the plant via direct contact with their roots (Kumar et al., 2018). These microorganisms are capable of colonizing the roots and facilitate the growth of plant along with controlling various stress either directly or by producing the phytohormones (Patel, 2018).

One common commercial product of PGPR is Indole-3-acetic acid (IAA). IAA is one of the characterized plant hormone, auxin. It is produced by plants, algae, mosses, lichens and other variety of microorganisms. This metabolite is procured from tryptophan (Trp) following both Tryptophan dependent and independent pathways by a wide variety of plants and micro-organism

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(Pattern and Glick, 2002).It plays major role in growth and development of plants. IAA has since been implicated in virtually all aspects of plant growth and development (Teale et al., 2006).

Besides its integral role in the growth and development of plants, PGPR has other beneficial usages that directly or indirectly improve growth. These benefits include control of other microorganisms, antagonistic activity, quorum sensing, signal interference, inhibitory action against biofilm formation, elevation in the level of mineral by solubilization mechanism, systemic acquired resistance and induced systemic resistance (Patten and Glick, 1996). Commercialization of products like biofertilizers obtained from PGPR (Adesemoye and Kloepper, 2009) has increased these days as it is turning out to be the better substitute of chemical fertilizers and pesticides which have various side effects on plants as well as the farmers (Banerjee et al., 2006). Therefore this study aims to identify such microorganisms from rhizospheric soil and assess their property to produce a product like IAA and quantify the amount of IAA produced with the help of sophisticated instruments like HPLC.

The agricultural productivity is being negatively affected by the unpredictable climatic changes that are the result of this rapidly growing population leading to higher anthropogenic activities which in turn brings numerous unwanted environmental stresses (Pereira, 2016). The agricultural production of this era is very less due to all these combined environmental stress and above all the need for maintain good food security for this growing population has led the focus towards determining the measures that could enhance and improve the agricultural productivity without destroying the natural biomass of the soil. Looking forward to the PGPR organisms would surely give the better way to overcome this issue effectively. Within this context the present study was designed for the isolation of the bacterial isolates capable of producing IAA and their characterization for their PGPR potential by the qualitative analysis. Moreover, the study includes the molecular characterization of the isolated with good IAA production ability by 16srRNA sequencing.

MATERIAL AND METHODS

Isolation of bacteria from rizospheric soil: Bacterial isolation was done from Rizospheric soil collected from the region around wheat plant roots. The soil was collected in zipped plastic bags and brought to the laboratory for isolation. The soil sample was serially diluted up to 10-8 dilution. 50 µl of the dilution (10-7) was spread on the freshly prepared nutrient plates. The plates were incubated at 37˚C for 48 hours and observation was recorded after completion of the incubation period. The colonies observed were streaked on different fresh plates for testing of various PGPR characteristics.

Characteristic PGPR production Assay Production of indole acetic acid (IAA): The detection of IAA by the isolates was done using Salkowski reagent (2% FeCl3 (0.5M), 35% perchloric acid) (Bric et al., 1991). The method could be briefly described as tryptic soy agar (TSA) media supplemented with 0.05% tryptophan was prepared and a membrane (nitrocellulose) was placed onto it. Suspension of the bacterial culture (20 µl) was added on the membrane and incubated at 28°C. This membrane was then placed on a filter paper soaked with Salkowski reagent. IAA production was detected by the development of pink halos near bacterial colonies.

Production of hydrogen cyanide (HCN): The detection of hydrogen cyanide (HCN) production by the bacterial isolates was done using sodium carbonate (2%) and picric acid solution (0.5%) (Bakker and Schippers, 1987). TSA media incorporated with 4.4 g/l glycine streaked with target isolates were sealed with paper inside it, the paper was earlier soaked with the above-mentioned reagent. Change in color to red indicates positive for HCN Production. Production of siderophores:Siderophore test was done using CAS (chrome azurol S) media (Schwyn and Neilands, 1987). The media was flooded with CAS medium at the top of TSA streaked with target microorganism. A positive test was indicated by an orange halo region around the streak.

Aminocyclopropane-1-carboxylate (ACC) deaminase activity: Aminocyclopropane-1-carboxylate deaminase activity was screened for target isolates on the sterile minimal DF (Dworkin and Foster) salts media with modification of 3 mM ACC added instead of (NH4)2SO4 (nitrogen source) (Dworkin and Foster, 1958; Penrose and Glick, 2003). Test samples were observed time to time for incubation period of 72 hours.

Molecular Characterization of isolates: DNA extraction for the isolates was done using kit Nucleo-pore gDNA Bacterial Mini Kit (Cat. NP-7006D). Quantification of the extracted DNA was performed using UV-Visible Double Beam Spectrophotometer (PC based Systronic 2202) observing absorbance at 260nm and 280nm. The quantification was recorded in ng/µl and purity ratio at 260/280nm was the record to estimate any contamination of protein or RNA. PCR amplification of 16S rRNA gene was done using universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1392R (5' TACGGTTACCTTGTTACGACTT 3') to identify species. PCR reactions for the detection of the bacterial species were set up with reactions mixture of 20 µl volumes containing 2 µl of the genomic DNA sample, 1× PCR buffer containing; 0.16 mM dNTP Mix; 20 pmol of forward and reverse primers and 0.75 U Taq DNA polymerase (MBI, Fermentas, Lithuania).

Amplification was carried out in a thermal cycler (Eppendorf Mastercycler) with the PCR conditions as follows: Initial denaturation at 95°C-6 min, denaturation at 95°C- 30 sec, annealing at 50°C-1 min, and extension at 72°C-1 min , final extension was performed at 72°C-10 min. Polymerase Chain Reaction was performed till 40 cycles. PCR products were analyzed using 1% agarose gel electrophoresis. The samples were then sequenced using Sanger's Method.

Quantification of IAA using HPLC Sample preparation:Sample preparation consisted of a single centrifugal filtration step using 3-kDa cut-off membrane centrifugal filters. For this purpose, 0.5 mL of bacterial culture supernatants or spiked sterile bacterial broths were transferred to the sample chamber of a 0.5 mL centrifugal filter tube and centrifuged at 14,000×g (relative centrifugal force) at 4 °C for 30 min. The filtrates were directly analyzed by HPLC

Instrumentation and chromatographic conditions: Eluent A consisted of 2.5: 97.5 % (v/v) acetic acid: H_2O, pH 3.8 (the pH was adjusted by addition of 1 mol L−1 KOH) and eluent B consisted of 80: 20 % (v/v) acetonitrile: H_2O. The mobile phase started with eluent A: eluent B at 80: 20 %, changing to 50: 50 %, 0: 100 % and 80: 20 % in 25, 31 and 33 min, respectively. The total run time was 20 min. The flow rate of the mobile phase was 1 mL min−1, the injection volumes were 20 µL, and the detector was set to excitation and emission wavelengths of 280 and 350 nm, respectively.

RESULTS AND DISCUSSION

Isolation of bacteria and screening for PGPR: Isolates obtained from serial dilution were then tested separately for PGPR activity. The results of the isolates are summarized in the table 1. In the two isolates positive results were observed as indication of the development of pink halos colonies on the addition of Salkowski's reagent. HCN production was observed for isolate 1 with the color change of the paper to red and no change in case of isolate 2 indicating a negative result. ACC deaminase was observed negative in both cases. Siderophore test was also observed positive in both Isolates tested on CAS media with an orange halo region around the test organism.

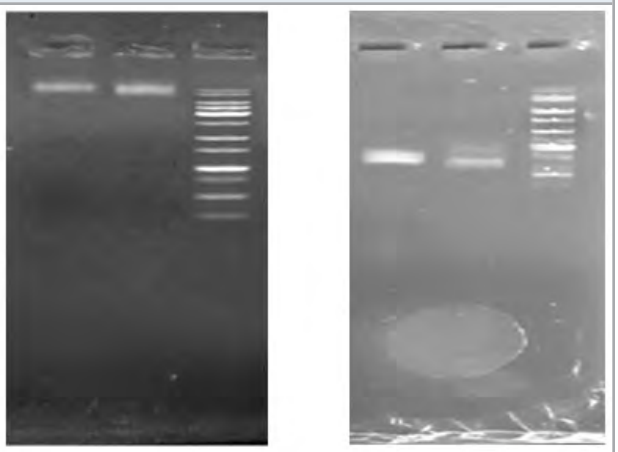
Table 1. Summarized result of PGPR for isolates

S.No.	Test	Isolate 1	Isolate 2
1	IAA Production	+	+
2	HCN production	+	-
3	ACC deaminase	-	-
4	Siderophore	+	+

*Positive result: (+) and Negative result: (-)

Molecular Characterization of Isolates: Molecular Characterization of two PGPR positive isolates was performed. The extracted DNA was observed on 0.1% agarose gel in the presence of UV light. The orange color bands confirmed the presence of DNA (Figure 1[A]). The concentration of the extracted DNA was determined using the spectrophotometric method. The purity ratio of 260/280 nm for Sample 1 and 2 were 1.809 and 1.689 respectively and the concentration of DNA calculated to be 7060 ng/µl and 6605 ng/µl. The concentration of DNA confirms its suitability for amplification, thus universal primers 27F and 1392R were used and PCR products of 1.4 kb -1.5kb amplicon size was observed as shown in figure 1[B].

Figure 1[A]: Genomic DNA at 0.8% Gel; Lane 1: Sample 1; Lane 2: sample 2 (bacterial sample); Lane 3: Ladder (1Kb) [B] PCR Product at 1.2 % Gel; Lane 3: Ladder (1Kb); Lane 1: Sample 1 (PCR Product); Lane 2:Sample 2 (PCR Product); Band Size: Approx 1.2-1.5 Kb



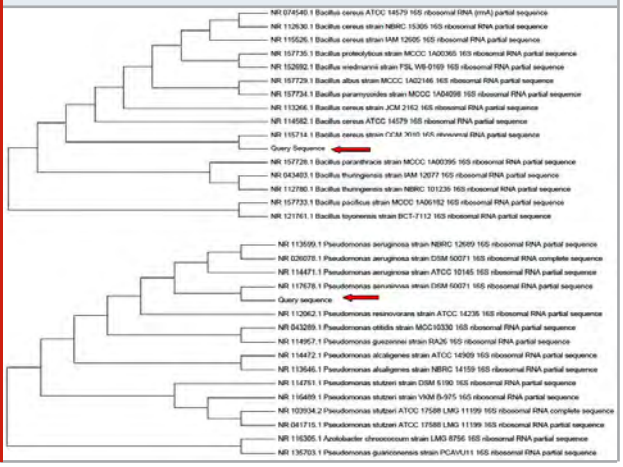
[A] [B]

The sequence obtained from sequencing was processed using a biological sequence alignment editor (BioEdit 7.2). The subsequent analysis had been done using NCBI-BLAST (National Centre for Biotechnology Information- Basic Local Alignment Search Tool) https://blast.ncbi.nlm.nih.gov/Blast.cgi and MEGA X (Molecular Evolutionary Genetics Analysis). The Sample 1 species was identified as *Bacillus cereus* as it was found 98.78% similar to *Bacillus cereus* strain CCM 2010 16S ribosomal RNA, partial sequence (NR_115714.1) (shown in figure 2). The phylogenetic tree constructed using the maximum parsimony method shows other closely related species. Sample 2 was identified as *P. aeruginosa* as it was found to be 99.23% similar to *Pseudomonas aeruginosa* strain DSM 50071 16S ribosomal RNA, partial sequence (NR_117678.1). Sequence submitted in the DNA Data Bank of Japan (DDBJ) with the accession number LC536053 and LC536054. The phylogenetic tree constructed using the maximum parsimony method shows other closely related species.

Quantification of IAA using HPLC: Earlier studies on Indole Acetic acid have revealed that it consist of acidic (IAA, ILA), amphoteric (Trp), basic (TAM) or essentially neutral (IAN, IAM, TOL) (Liu et al., 2019). Thus selection of pH range for mobile phase plays an important role in proper separation, retention time and peak shape of ionizable compounds (Espinosa et al., 2002; Chandrul and Srivastava, 2010). Here, a gradient for mobile phase was chosen and proportions of various solvents were set accordingly to obtain better separation. The selected eluents: Eluent A consisted of 2.5: 97.5 % (v/v) acetic acid: H_2O, pH 3.8 (the pH was adjusted by addition of 1

mol L–1 KOH) and eluent B consisted of 80: 20 % (v/v) acetonitrile: H_2O.

Figure 2: Phylogenetic Tree constructed using MEGA X by Maximum Parsimony method [A] Sample 1 [B] Sample

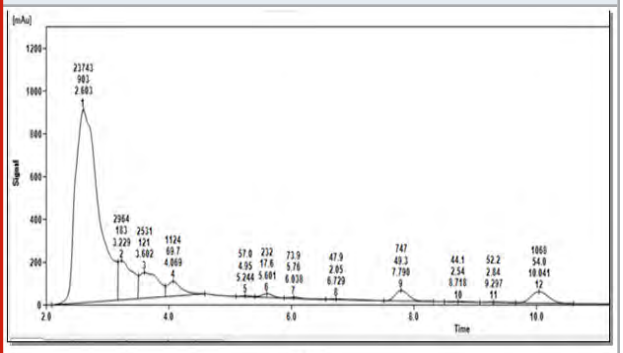


The mobile phase started with eluent A: eluent B at 80: 20 %, changing to 50: 50 %, 0: 100 % and 80: 20 % in 25, 31 and 33 min, respectively. The total run time was 20 min. The flow rate of the mobile phase was 1 mL min–1, the injection volumes were 20 µL, and the detector was set to excitation and emission wavelengths of 280 and 350 nm, respectively. Thus, following above mentioned conditions, 7 major well separated peaks, but not symmetrical, were observed in each sample. Retention times (min) of the peaks were 2.603, 3.229, 3.602, 4.069, 5.601, 7.790, 10.041 and 2.224, 2.846, 3.183, 3.676, 5.226, 7.262, 9.478 for sample 1 and sample 2 respectively were observed.

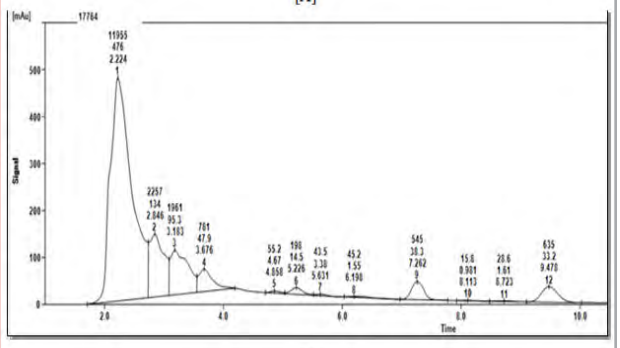
The linearity and range of the analysis was assessed by preparation and analysis of different concentrations of the standard solutions of the IAA. Calibration curves of standards were plotted for the stock solutions. The range of concentration for IAA standard was kept between 0.05µg/ml-0.025 µg/ml. Our results showed presence of numerous indolic compounds which was in accordance to work discussed by different authors Ahmad et al. (2005), Khakipouret al. (2008) and Chaiharn and Lumyong (2011).Thus bacterial supernatant have presence of load of such compounds, Production of such compounds by bacterial species does not require supplemented enriching compounds. And as mentioned in Szkop and Bielawski (2013) centrifugal filtration method was followed to reduce analytes with high molecular weight (> 3 kDa), our study followed to remove contaminants at initial stage.

This also ensures purity of the sample with less numbers of noise and detectable compounds in chromatogram. The aim of this study was to estimate qualitatively and Quantitatively IAA production by rhizospheric soil microbes. Several strains of genus *Bacillus, Azotobacter, Pseudomonas* were reported to produceIAA (Cassán et al., 2014; Verma et al., 2018). The evaluationof bacterial isolates for production of IAA have revealed that both are significant producers of IAA hence they could be used as PGPR.The identified bcaterial species were B.cereus and P.aeruginosa. IAA production was quantified by HPLC technique and both isolates showed comparable quantity of IAA in the supernatant. Earlier studies have enlightened the role of IAA produced by rhizospheric soil microbes (Wahyudiet al., 2011).

Figure 3: Chromatograms obtained in the analysis of IAA [A] Chromatogram of B.cereus culture supernatants after 72 hrs incubation period in King B medium supplemented with 3.5 mM Trp. [B] Chromatogram of P.aeruginosa culture supernatants after 72 hrs incubation period in King B medium supplemented with 3.5 mM Trp.



[A]



[B]

Some reported species for IAA production, root elongation in *Sesbaniaaculeata* by inoculation with *Azotobacter* spp. and *Pseudomonas* spp., in *Brassica campestris* by *Bacillusspp* (Ghosh et al., 2003), in Vigna radiate by *Pseudomonas putida* (Pattern and Glick, 2002) and in *Pennisetumamericanum* by *Azospirillumbrasilense*(Tien et al., 1979).Effect of IAA producing isolate was also observed in *Solanum lycopersicum*, (Khan et al., 2016) where it significantly increased the shoot and root biomass and chlorophyll (a and b) contents as compared to control plants.Plant roots secrete tryptophan in the rhizosphere which is utilized by rhizobacteria as a precursor for IAA biosynthesis (Shameer & Prasad, 2018).The IAA producing bacteria are known to assist the plant growth and they can even effectively protect them from the various environmental stress inclusing the salinity stress as reported the IAA producing microrganism promote plant growth both under normal and saline conditions (Gupta and Pandey, 2019; Kang et al., 2019).

CONCLUSION

PGPR is being studied by researchers to explore products that are yet unexplored or to identify sources of microbes with a maximum yield of these products. The microbes that colonize the roots of the plants or are freely available in the soil can produce a wide range of products that contributes to plant growth promotion, biocontrol agents, disease resistance agents, insecticidal effect and many more. We aimed to isolate and characterize such PGPR isolates and emphasize on their IAA producing capability. The quantity of IAA was also estimated using the technique of HPLC. This method could prove as a tool to quantify IAA or other PGPRs between different bacterial species.

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Toxicological Communication

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Impact of Arsenic on Biochemical Components of *Abelmoschus esculentus*

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ABSTRACT

The study revealed the impact of various concentration of arsenic chloride on the biochemical characteristics of *Abelmoschus esculentus* L. The results have shown that photosynthetic pigment such as total chlorophyll shows declines trend form stress of 2mM to 10mM. The total soluble sugar and the protein content in the leaves were found to decrease with increase in the short concentration of heavy metal treatment. The most abundant forms of arsenic in the environment are the inorganic As (V) and As (III) species, and only the organic species monomethyl arsenic acid (MMAA) and dimethyl arsenic acid (DMAA) can be found in detectable concentrations. The form in which As is present in the environment influences its chemical behaviour and its toxicity. Concentrations of arsenic in uncontaminated soils range from 0.2 to 40 ppm. In uncontaminated soils, the level of arsenic is not sufficiently high to cause phytotoxicity and does not, therefore, represent a health hazard. Soils from land repeatedly treated with inorganic arsenicals contain levels of arsenic often 10-100 folds those of untreated areas. Use of arsenicals as insecticides usually results in higher concentrations of arsenic in the soil than when they are used as defoliants. Under strongly reducing conditions elemental arsenic (AS_4) and arsine (AsH_3) (-III) are the stable forms. Species in the cells causing cell damage. Research studies revealed that arsenic can exert its toxic effects through impairment of cellular respiration by inhibition of various mitochondrial enzymes and uncoupling of oxidative phosphorylation. The As (III) species can react with the SH group of protein and enzymes, thereby make them inactive and increase reactive oxygen enzymes in the body..

KEY WORDS: ABELMOSCHUS ESCLULENTUS, ARSENIC CHLORIDE, MMAA (MONOMETHYL ARSENIC CHLORIDE), DMAA (DIMETHYL ARSENIC ACID).

ARTICLE INFORMATION

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INTRODUCTION

Arsenic pollution in groundwater has become a major public concern in many countries and potentially impacting millions of people since more and more groundwater withdrawal are taking place due to human usage and agricultural irrigation. The most abundant forms of arsenic in the environment are the inorganic As (V) and As (III) species, and only the organic species monomethyl arsenic acid (MMAA) and dimethyl arsenic acid (DMAA) can be found in detectable concentrations (Rahman et al., 2008; Tlustos et al., 2002). The form in which As is present in the environment influences its chemical behaviour and its toxicity. Generally, As (III) is more mobile and more toxic than As (V) and inorganic arsenicals are more toxic than organic arsenicals (Chung et al., 2006; Inskeep et al., 2002, Chakarborti et al., 2018). Concentrations of arsenic in uncontaminated soils range from 0.2 to 40 ppm. In uncontaminated soils, the level of arsenic is not sufficiently high to cause phytotoxicity and does not, therefore, represent a health hazard.

Soils from land repeatedly treated with inorganic arsenical contain levels of arsenic often 10-100 folds those of untreated areas. Use of arsenicals as insecticides usually results in higher concentrations of arsenic in the soil than when they are used as defoliants. Under strongly reducing conditions elemental arsenic (AS_4) and arsine (AsH_3) (-III) are the stable forms (Wan et al., 2019). In a less reduced environment such as those in flooded soils, the relatively toxic arsenite ($MAsO_2$) (+III) can be formed. However, in aerated soils, arsenate ($MAsO_4$) (+V) predominates. In reduced through arsenite to dimethyl arsenic acid, which is extremely toxic. Soluble arsenic has been observed to increase in flooded rice soils (Wan et al., 2019). Arsenic can exert its toxic effects through impairment of cellular respiration by inhibition of various mitochondrial enzymes and uncoupling of oxidative phosphorylation. The As (III) species can react with the SH group of protein and enzymes, thereby make them inactive and increase reactive oxygen species in the cells causing cell damage. Research studies revealed that Arsenic could inhibit 200 enzymes in the body. It has been regarded that multi-systemic non-cancerous could be due to deactivation of essential enzymatic functions by trivalent Arsenic compounds and subsequent oxidative stress to cell (Wan et al., 2019).

More recent studies have detected all the four species [As (III), As (V), MMA (V), DMA (V)] and also the presence of MMA (III) and DMA (III) in the urine. It is also considered that inorganic As (III) and the reduced forms of MMA (III) and DMA (III) formed during methylation are highly reactive and contribute to the observed toxicity of inorganic Arsenic. So far, no evidence has been found that inorganic Arsenic directly causes genetic mutations affecting cancerous cells. Inorganic Arsenic indirectly enhances susceptibility to cancer-inducing chromosomal alterations, inhibition of DNA repair process, oxidative stress and cell proliferation. Arsenate (AsO_4^{3-}) has a similar structure as phosphate (PO_4^{3-}) and thus can substitute for PO_4^{3-} in adenosine diphosphate (ADP). This substitution prevents the conversion of ADP to ATP which produces energy to the cell (Wan et al., 2019).

MATERIAL AND METHODS

Collection of Plant Material: Seeds of *Abelmoschus esculentus*, L., was procured from local seed centre, Patna, Bihar, India.

Abelmoschus esculentus, L. Var. S7 (Family; Malvaceae) was chosen as an experimental plant. The effects of various concentrations of arsenic chloride on biochemical features were analyzed on the selected plant.

Preparation of the Experimental Soil: The experimental soil for raising the cultivars was prepared by mixing red soil, black soil and sandy soil in the ratio of 1:1:1. The prepared soil was sterilized by solar sterilization method for 5 days (Handiseni et al., 2010). It was then analysed for its physicochemical properties. The analysed soil medium was taken in earthen pots of size 30×33 cm and filled in about two-third of its height (5 kg of soil per pot).

Heavy Metals used for the Study: To investigate the effect of heavy metals on *Abelmoschus esculentus* L., Arsenic was applied in the form of arsenic chloride ($AsCl_2$).

Experimental Design:

Heavy Metals Stress on *Abelmoschus esculentus*: The heavy metal arsenic was treated separately in the experimental plant with different concentrations viz., 2 mM, 4 mM, 6 mM, 8 mM and 10 mM (w/v) in five replicates. The aqueous solutions of heavy metal were applied to the soil after the development of first leaves in the seedlings. Then the plant was watered with the respective concentration of metals on every alternate day. A set of plants without heavy metal treatment was maintained as control. The surface-sterilized seeds of *Abelmoschus esculentus*, L., was sown uniformly in the pots for the experimental purpose. The Biochemical parameters and metal concentration in plants were analysed on the 35th day after planting (DAP).

Impact assessment of Arsenic chloride on Biochemical characteristics:

Estimation of Chlorophyll: For extracting total chlorophyll from leaves, fresh leaves were deveined and cut into small bits. From the pooled leaf bits, a sample of 100 mg was weighed. The leaf bits were homogenized in 100% acetone using a mortar and pestle. The homogenate was centrifuged at 4000 rpm for 5 minutes at room temperature. Extraction with 100% acetone was repeated till the pellet becomes pale-yellow or white. The supernatant was used for the estimation of photosynthetic pigments. The absorbance was measured at 662nm, 645nm and 470nm for chlorophyll a, b and carotenoids, respectively using a spectrophotometer. The amount of chlorophyll a, chlorophyll b and total chlorophyll was calculated using the formulae of Wellburn and Lichtenthaler 1984.

$$\text{Chlorophyll } a\,(\text{mg/L}) = 11.75 \times A_{662} - 2.35 \times A_{645}$$

$$\text{Chlorophyll } b\,(\text{mg / L}) = 18.61 \times A_{645} - 3.96 \times A_{662}$$

$$\text{Total chlorophyll} = \text{chlorophyll } a + \text{chlorophyll } b.$$

Estimation of Total Soluble Sugar: Exactly 100 mg of leaf sample was ground in 20 mL of distilled water using a mortar and pestle. The homogenate was filtered through two layers of cheesecloth and the filtrate was spun at 3000 rpm for 5 minutes. The pellet was discarded and the supernatant was taken. Three mL of Trichloroacetic acid (TCA) was added to the supernatant. It was thoroughly mixed and kept in ice for 10 minutes. This mixture was centrifuged at 3000 rpm for 5 minutes. The pellet was discarded and the supernatant was used as a test solution. An aliquot of 0.1 mL of test solution was taken in a test tube and to this, 0.9 mL of distilled water and 4 mL of anthrone reagent were added. The solution was mixed thoroughly and the tubes were kept in boiling water for 10 minutes. Glucose content was measured using a standard value (Jayaraman, 1981).

Estimation of Protein: The total soluble protein was estimated by Lowry's method (Lowry et al., 1951). Fresh leaf samples were ground in 10 mL of distilled water using mortar and pestle. The homogenate was spun at 3000 rpm for 5 minutes. The supernatant was taken and the pellet was discarded. To the supernatant, 1 mL of ice-cold 10% (w/v) TCA was added and kept in ice for 10 minutes. The extract was centrifuged at 5000 rpm for 10 minutes. The pellet was dissolved in 0.1N NaOH and used as the test solution.

Reagents for Protein Estimation

A) 0.5% of $CuSO_4$: Solution A

B) 1% Sodium-Potassium tartarate: Solution B

C) 2% Na_2CO_3 solution in 0.1 N NaOH: Solution C

The mixture of 0.5 mL of A and 0.5 mL of B with 4.9 mL of solution C is known as an alkaline copper reagent. An aliquot of 0.1 mL of test solution was taken in a test tube and 0.4 mL of distilled water, 0.5 mL of freshly diluted (1:1) folin-phenol reagent and 5.5mL of alkaline copper reagent were added. Contents in the test tube were mixed immediately and left undisturbed for 10 minutes for the development of blue colour. The absorbance was measured at 650 nm with a spectrophotometer with alkaline copper reagent as blank. The protein content was calculated from a standard graph of protein constructed with bovine serum albumin (BSA) as marker protein.

RESULTS AND DISCUSSION

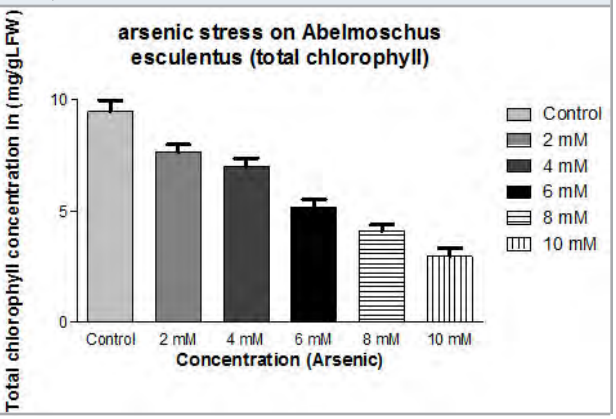
Total Chlorophyll Content: The results of the effect of arsenic chloride and nickel chloride on the photosynthetic pigment contents of co-cultivated *Abelmoschus esculentus*, L. have been represented in Table 1. The total chlorophyll content under arsenic chloride treatment was found reduced to 19% (at 02 mM Conc.; Mean± SD 7.65± 0.311) to 69% (at 10 mM Conc.; Mean± SD 2.95±0.364) in *Abelmoschus esculentus*, L.

Table. 1

Arsenic stress on *Abelmoschus esculentus* (Total Chlorophyll)

Sample	mean±SD	%Change
Control	9.45±0.492	
2mM	7.65±0.311	-19.0476
4mM	6.99±0.354	-26.0317
6mM	5.16±0.359	-45.3968
8mM	4.11±0.261	-56.5079
10mM	2.95±0.364	-68.7831

Figure 1: Impact of arsenic chloride on the total chlorophyll content (mg/gLFW) of (*Abelmoschus esculentus* L.) ($p \leq 0.05$)



Total Soluble Sugar: The reduction in total Soluble Sugar and arsenic chloride treatment in *Abelmoschus esculentus* was 18% (at 02 mM Conc.; Mean± SD 6.278±0.394) to 52% (at 10 mM Conc.; Mean± SD 3.638± 0.233) represented in table 2.

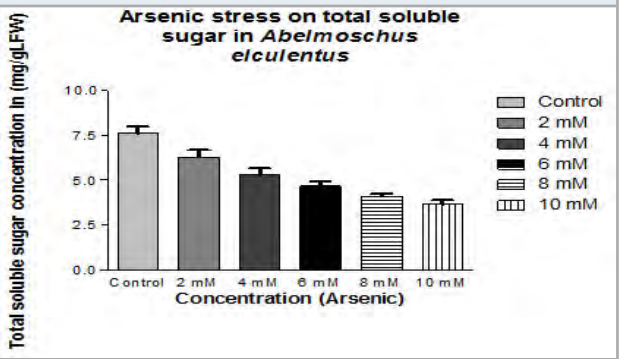
Table. 2

Arsenic stress on Total Soluble Sugar in A. E

Sample	mean±SD	%Change
Control	7.60±0.393	
2mM	6.278±0.394	-17.5
4mM	5.270±0.389	-30.65789474
6mM	4.652±0.270	-38.81578947
8mM	4.082±0.144	-46.31578947
10mM	3.638±0.233	-52.23684211

mM Conc.; Mean± SD 3.638± 0.233) represented in table 2.

Figure 2: Impact of arsenic chloride on the total soluble sugar content (mg/gLFW) of (*Abelmoschus esculentus* L.) ($p \leq 0.05$)



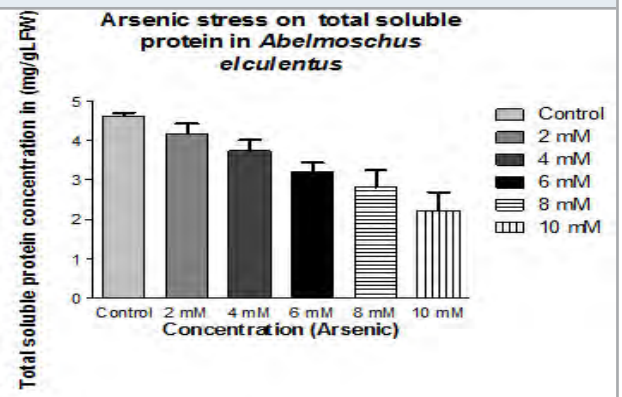
Total Soluble Protein: Under Arsenic Chloride treatment the percentage change in same plant was 09% (at 02 mM Conc.; Mean± SD 4.19± 0.244) to 52% (at 10 mM Conc.; Mean± SD 2.22± 0.461) represented in table: - 3

Table. 3

Arsenic stress on total soluble Protein in A. E

Sample	mean±SD	%Change
Control	4.62±0.0873	
2mM	4.19±0.244	-9.307359307
4mM	3.74±0.276	-19.04761905
6mM	3.20±0.248	-30.73593074
8mM	2.82±0.432	-38.96103896
10mM	2.22±0.461	-51.94805195

Figure 3: Impact of arsenic chloride on the total protein content (mg/gLFW) of (*Abelmoschus esculentus* L.) ($p \leq 0.05$)



Discussion: Inhibition of the photosynthetic pigment biosynthesis is one of the primary events in plants during heavy metal stress (Prasad and Prasad 1987). As a consequence, a delay in the assembly of the photosynthetic apparatus, lower photosynthetic efficiency, slower plant growth and decreased biomass production occur. Thus, heavy metal pollution could be a serious agricultural problem as it decreases the yield of crop plants and lowers the quality of plant products due to increased content of toxic metals (Melo et al. 2009). The present study revealed the impact of various concentrations of arsenic on the biochemical characteristics, of *Abelmoschus esculentus*, L. where photosynthetic pigments such as chlorophyll and carotenoid also showed a similar significant declining trend. However, the Anthocyanin accumulates in the leaves with an increase in the concentration of metal indicating its antioxidant property and protective function against heavy metal pollutants (Melo et al. 2009).

The total soluble sugar and the protein content in the leaves were found to decrease with the increase in the concentration of heavy metals treatment. Reduction in protein level can be directly correlated to the observed increase in the accumulation of free amino acids. Proline accumulation was more in the stressed plants than in the control. Under the heavy metal treatment, there was a considerable reduction in the growth and photosynthetic pigments, which could be due to the disturbance in photosystem I and induced activity of chlorophyllase enzyme. This disturbance paralleled with the reduction in sugar content could be attributed to the reduction in chlorophyll contents of the leaf and also a decline in protein. This change might have already affected the photosynthetic activity in the plant and hence the reduction in carbohydrate contents (Dowton, 1977; Swaminathan et al., 1998).

The major total soluble protein in the leaf is RUBPCase. A reduction in leaf protein indicated the reduction in RUBPCase, which caused a reduction in photosynthetic activity, which in turn, affects the total soluble sugar level (Goodwin and Mercer, 2005). Reduction in the protein contents in the roots, leaves and petioles of water hyacinth and lettuce plants after chromium treatment and suggested that metal ions seem to interfere with protein synthesis which is one of the major components of biochemical activities. In the present study, a reduction in protein content observed in both arsenic and nickel treated plants, could be attributed to the decrease in the synthesis of protein macromolecules under metal toxicity and the denaturation of protein by protease activity resulting in increasing level of protein degradation. The reduction in sugar content could be attributed to the reduction in chlorophyll content of the leaf and also a decline in protein. This change might have already affected the photosynthetic activity of the plant and hence the reduction in carbohydrate content (Macfie and Taylor, 1992).

Impaired carbon flow through the glycolytic pathway may sometimes due to disturbed carbohydrate metabolism which decreases the rate of sucrose formation as reported (Clemens, 2006). As a result of protein degradation, the availability of free amino acids is significantly high in hyperaccumulators. The free amino acid content is increased with increasing concentration of the arsenic chloride and nickel chloride. It may be due to the destruction of protein or increase in the biosynthesis

of amino acids from the nitrate source, which was not utilized in the protein synthesis (Schmoger et al., 2000). The degradation of protein may lead to an increase in free amino acid content. It is an adaptive mechanism employed by the plant cell to overcome post-stress metabolism (Singh and Vijayakumar, 1974). Our work emphasized that to science-based decisions with standards and limitations set by regulatory bodies and a clearer understanding and explanation of observation on wastewater treatment systems.

Summary: The present study revealed the impact of various concentrations of arsenic on the biochemical characteristics, of *Abelmoschus esculentus*, L. Photosynthetic pigments such as chlorophyll and carotenoid also showed a similar significant declining trend. However, the anthocyanin accumulates in the leaves with an increase in the concentration of metal indicating its antioxidant property and protective function against heavy metal pollutants. The total soluble sugar and the protein content in the leaves were found to decrease with the increase in the concentration of heavy metals treatment. Reduction in protein level can be directly correlated to the observed increase in the accumulation of free amino acids. Proline accumulation was more in the stressed plants than in the control.

CONCLUSION

Extensive progress has been made in characterizing soil chemistry management needed for phytoremediation, and physiology of plants which hyperaccumulate and hypertolerate metals, there is still the need for more nutrient-related research monitoring to achieve unpolluted wastewater discharge. It is increasingly clear that hypertolerance is fundamental to hyperaccumulation, and high rates of uptake and translocation are observed in hyperaccumulator plants. Search for superior hyperaccumulator plants and agronomic technology to improve the annual rate of phytoextraction and to allow recycling of water toxic metals accumulated in plant biomass is very likely to support commercial environmental remediation which society can afford. In addition, opportunities should be identified for research and development to improve the efficiency of phytoremediation. This will help to ensure science-based decisions with standards and limitations set by regulatory bodies and a clearer understanding and explanation of observation on wastewater treatment systems.

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Nanotechnological Communication

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Eco-Friendly Synthesis of Silver Nanoparticles Using *V. serpens* Plant and Evaluation of their Antibacterial Activity Against *Enterococcus faecalis*

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ABSTRACT

An attempt was made to synthesize biocompatible silver nanoparticles by using V. serpens plant extract, which can be used in different biomedical applications. The synthesis of silver nanoparticles was optimized in various physicochemical conditions and highly stable silver nanoparticles were synthesized with 8.0 mL of *V. serpens* leaf extract, pH 7.0, 1.0 mM $AgNO_3$ and 37 °C. The synthesized silver nanoparticles were characterized by UV-vis spectrophotometer, Ph analysis, FE-SEM, XRD and FTIR analysis. The average size of synthesized silver nanoparticles was 60-70 nm. The findings from our study showed that the aqueous extract of V. serpens and silver nitrate solution did not show significant inhibiting activity against *Enterococcus faecalis* bacteria in tested concentrations but the green synthesized silver nanoparticles, inhibited the growth of *Enterococcus faecalis,* which is responsible for many human diseases like urinary tract infection (UTI). In addition, synthesized silver nanoparticles showed a synergetic effect on the antimicrobial activity of the standard antibiotic levofloxacin against *Enterococcus faecalis* bacteria under this study. These findings indicate that green synthesized silver nanoparticles can be used to control *Enterococcus faecalis*-related human diseases as effective growth inhibitors.

KEY WORDS: SILVER NANOPARTICLES; ANTIBACTERIAL ACTIVITY; SCANNING ELECTRON MICROSCOPY, FTIR, XRD.

INTRODUCTION

Research and development in nanotechnology are a fast-growing field worldwide (Ahmed et al., 2016). Nanobiotechnology is currently one of the vibrant research disciplines of contemporary material sciences in which plant and plant products find an imperative use in nanoparticles synthesis (Banerjee et al., 2014; Terra et al., 2019). Such particles exhibit entirely new characteristics such as size scale, morphology and distribution compared to the larger particles of the mass material from which they were prepared (Raju et al., 2013; Elemike et al., 2020). Silver nanoparticles (AgNPs), with a broad number of applications including its antibacterial catalytic properties and its non-toxicity towards human health, are one of the most commonly used varieties of NPs (Amooaghaie et al., 2015; Ferdous et al., 2020).

Genes that couple with silver nanoparticles can inhibit the progression of the cell cycle, DNA damage and apoptosis in human cells at non-cytotoxic doses

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(Asharani et al., 2009; Carvalho et al., 2018). Mechanisms for toxicity caused by AgNP include genetic material, mitochondria and cell membranes. As the silver ions enter the bacterial cell, the DNA molecule is condensed and loses its ability to replicate causing bacterial cell death (Feng et al., 2000; Rajoka et al., 2020). Many techniques for synthesizing AgNPs, including physical, chemical and, most recently, biological methods have been investigated Nanoparticles are quickly synthesized in chemical synthesis which uses harmful chemical materials such as sodium borohydride, hydroxylamine, hydrazine, and ethanol (Negm et al., 2015, Amooaghaie et al., 2015; Bilal et al., 2019).

In addition, the aggregation, growth, and stability of particles are difficult to control with chemical synthesis and capping agents are required for the stabilization of nanoparticles size (Saha et al., 2017). Recently great attention has been paid to green synthesis using different parts of plant extracts for nanoparticles synthesis (Bilal et al., 2017; Hemmati et al., 2019). Biosynthetic green metal nanoparticles are increasingly being used as a result of their usability, non-toxicity and availability for large-scale processing (Nagajyothi et al., 2012; Sharma et al., 2019). This method is superior to traditional synthesizing because it is an environmentally sustainable, inexpensive, single-step process, and simple to apply to large-scale manufacturing and also does not require high pressures, temperature, resources or toxic chemicals. Much research has been done on the green synthesis of silver nanoparticles using bacteria, fungi and various plant extracts; due to their antioxidant properties, they are able to reduce metal compounds to their respective nanoparticles (Omidi et al., 2018; Manosalva et al., 2019).

Plant extracts provide the best capping content for silver nanoparticles' stabilization (Ahmed et al., 2016; Alfuraydi et al., 2019). Medicinal plants have been used in India since ancient times for their ability to function against both infectious and non-infectious diseases and have been properly referred to in Ayurveda. Traditional medical practitioners also increased the use of medicinal plants for the treatment of various diseases (Azaizeh et al., 2003). Viola serpens is a plant belonging to the Violaceae family. For preparation of antibacterial medicinal plant extract, the different sections of different viola species are used. Several species within the Violaceae family are rich in cyclotides and contain phytochemical products (Gerlach et al., 2010; Chand et al., 2019). The biological synthesis of silver nanoparticles has yet to show bioreduction mechanisms through the use of *V. serpens* extract, though this plant is commonly used in medical usage (Gerlach et al., 2010; Chand et al., 2019).

The *V. serpens* extract as a bioreduction reducer which transforms silver ions into silver nanoparticles has been shown in this study. Biologically synthesized silver nanoparticles were characterized by UV-vis spectroscopy, Field emission scanning electron microscopy (FE-SEM), Fourier transform infrared spectroscopy and X-ray diffraction techniques (Chand et al., 2019). The present investigation notes the simple green synthesis of AgNPs using *V. serpens* leaf extracts and their anti-microbial action against *Enterococcus faecalis*. The method of well diffusion has been used to determine the antibacterial ability of green synthesized AgNPs (Chand et al., 2019).

MATERIAL AND METHODS

Collection of plant materials and preparation of extract: In August 2019, *V. S erpens* fresh mature leaves were collected from the Dehradun hilly region for plant extract preparations, green synthesis of silver nano particles and antibacterial analysis of green synthesized silver nanoparticles. The plant leaves were then washed carefully three times with water, followed by distilled water to remove dirt and dust on the leaf surface. These leaves were then dried and finely made powder with pestle and morter. Until the extraction, in a separate case, the powdered product was stored. Weighed and mixed 10 g of powdered plant material with 80 mL of deionized water and boiled it for 20 minutes. Then the material was filtered through the paper filter (Whatman No. 1). For further investigations, the prepared extract was kept at 4 °C.

Synthesis of silver nanoparticles by using the *V. serpens* extract: *V. serpens* extract was used for the synthesis of silver nanoparticles. In order to achieve the best values, it was studied the effects of the extract quantity and the silver nitrate solution concentration. By adjusting the amount of extract, the best concentration was achieved. The different concentration levels of silver nitrate solutions were then added to different amounts of 1-15 mL extract. The plant extract volume of 8 ml and silver nitrate solution concentration of 1 mmol / L were both optimally chosen. Drop by drop, eight mL plant extracts have been applied in 1 mmol / L silver nitrate solution for 40 mL. Colorless to dark gray, the silver nitrate solution revealed the development of silver nanoparticles. The solution containing silver nanoparticles was centrifuged to removal of silver nanoparticles from the other composition of a solution at 9000 RPM for 15 minutes and the deposit was prepared for the appropriate analysis.

Characterization of green synthesized silver nanoparticles: Silver nanoparticles can be characterized in a number of ways. The first and most common approach is the color changing of the solution. We used the Fourier transform infrared spectroscopy and UV-vis spectrophotometer for determination of optical properties of green synthesized silver nanoparticles. A Wensar UV-vis spectrophotometer was used for UV-vis spectral analysis and at a 300 nm / min scan speed the test sample had been scanned between 200 to800 nm. As a blank point of reference was the deionised water.

Initially pH 3.9 was recorded for 1 mmol/L aqueous solution of silver nitrate and pH changes were observed to indicate the synthesis of silver nanoparticles with plant extracts. Digital pH meter of Wensar were used to

determine the pH. A high-resolution FE-SEM analysis was used for the morphological characterization of the synthesis of silver nanoparticles. The sample was made using a simple drop cover with a synthesized silver nanoparticle suspension in a clean electric glass to evaporate the solvent. At room temperature, the sample was allowed to dry. With the use of phase scan and Cu-Ka radiation (1,500 Å, 40 kV, 30 mA) in 1-2 h, X-Pert Pro diffractometer obtained the results for x-ray diffraction. The glass slide was filled with samples of biosynthesised silver nanoparticles accompanied by drying and an X-ray diffractometer eventually examined.

Antibacterial assays of synthesized silver nanoparticles: Agar well diffusion test was used to examine the antimicrobial activity of green synthesized silver nanoparticles. With the help of sterile cotton swab the tested bacteria were swabbed uniformly on two nutrient agar plates, then wells of 6 mm diameter were created by using sterile cork borer. In one plate the corresponding well has been covered by twenty-five microliters of synthesized silver nanoparticles with varying concentration (1.0 and 2.0 mM) and in other plate well has been covered by silver nitrate solution as control and plant extract. In order to determine the combined effect of standard antibiotic (levofloxacin) and the synthesized silver nanoparticles, 15 mL of 0.5 mM antibiotic solution were mixed with 15 mL of synthesized silver nanoparticle solution (1 mM) and placed with the microorganisms tested (*Enterococcus faecalis*) in the corresponding agar plate well. The plates were then incubated for 24 h at 370C. The inhibition zone of synthesized silver nanoparticles, plant extract, silver nitrate solution, levofloxacin and synthesized silver nanoparticles with levofloxacin antibiotic was measured and compared individually.

RESULTS AND DISCUSSION

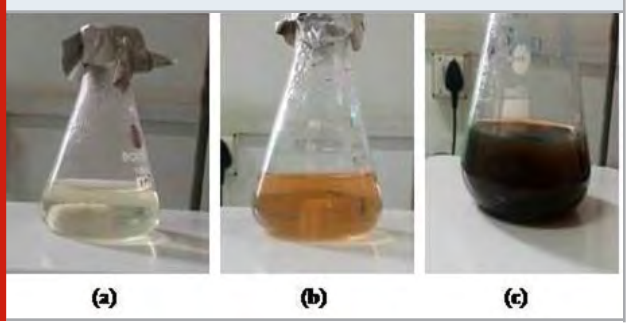
The biological and environmental safety of the production process in many sectors of the economy is becoming an increasing concern while metal nanoparticles are being more and more used. Key methods for production of nanoparticles are chemical and physical strategies that are costly, and potentially environmentally damaging. The risks to human health are getting more severe with antimicrobial and antibiotic resistances. It must be overcome with nature's support. Because of the urgent need for antimicrobial drugs, research on the chemistry of medicinal plants is increasingly concerned (Meenambigai et al., 2018).

Medicinal plants have been a potentially therapeutic source for thousands of years. There are a significant number of modern medicines coming from natural sources such as plants which are known for thousands of years as part of the advancement of human health care (Patil et al., 2019). In the past decade, furthermore, many organic systems such as plants have been shown to transform metal ions into metal nanoparticles through their reductive metabolite capacity (Usmani et al., 2019). Silver has been widely used in human culture for many years. It is the only element that can be tuned with its plasmon resonance at any wavelength of the visible spectrum. This element has a large number of applications, including its disinfectant property. Silver nanoparticles have shown antibacterial properties with a close connection to the microbial cell of the nanoparticles and activity based on their dimension (Ahmad et al., 2019; Fahimmunisha et al., 2020).

Basic studies showed the valuable property of silver nanoparticles, namely the special optical characteristics of catalytic operations, resonance of surface plasmon, high electric double-layer efficiency and so on, are rarely combined. Nano silver particles were commonly used as an antibacterial agent in the healthcare industry, for preserving food, cloth coatings and in specific environmental applications (Thomas et al., 2019). In this respect the synthesis of silver nanoparticles by V. serpens extracts was carried out using an efficient and versatile process. In order to achieve the best values, it was studied the effects of the extract quantity and the silver nitrate solution concentration. By adjusting the amount of extract, the best concentration was achieved. Drop by drop, plant extracts have been applied in 1 mmol / L silver nitrate solution. Colorless to dark gray, the silver nitrate solution revealed the development of silver nanoparticles. The solution containing silver nanoparticles was centrifuged to removal of silver nanoparticles from the other composition of a solution at 9000 RPM for 15 minutes (Thomas et al., 2019).

Then the deposit was prepared for the appropriate analysis. For the detection of the optical properties of silver nanoparticles, we used UV-vis and FTIR spectrums. The surface plasmon resonance of biosynthesized silver nanoparticles displayed a peak at the UV-vise range of almost 430 nm, which correlates to the absorbance of silver nanoparticles. We compared the antibacterial properties of biosynthesized silver nanoparticles, aqueous *V. serpens* sample extract, standard antibiotic levofloxacin and biosynthesized silver nanoparticles with biosynthesized silver nanoparticles (Fahimmunisha et al., 2020).

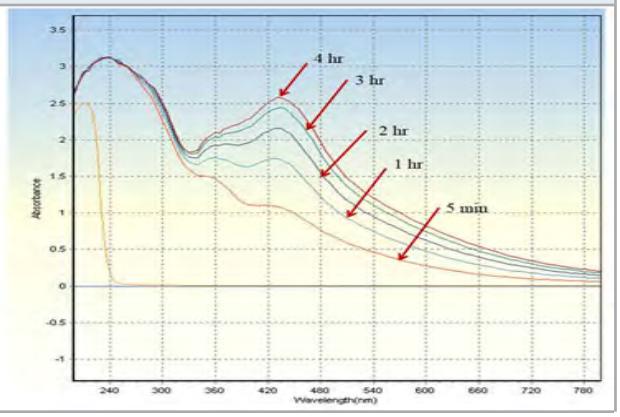
Figure 1. Synthesis of silver nanoparticles by using *V. serpens* plant extract (a) Solution of silver nitrate and *V. serpens* plant extract (after 2min); (b) Solution of silver nitrate and *V. serpens* plant extract (after 15 min); (c) Solution of silver nitrate and *V. serpens* plant extract (after 45 min).



Colour change and UV–vis spectroscopy: The primary indication of the formation of silver nanoparticles is the color change of the reaction solution to dark brown (Behravan et al., 2019). Addition of silver nitrate to V. serpens leaf extract produced and instantaneous colour change from an initial, light yellow solution to dark brown solution within 45 min of reaction time (Figure 1) (Fahimmunisha et al., 2020).

The silver nanoparticles surface plasmon resonance showed a peak centered close to 430 nm which corresponds to the absorption of silver nanoparticles (Figure 2) (Roy et al., 2019). The intensity of the reaction time increased until the fourth hour without a change in the peak wavelength, as illustrated in Figure 2.

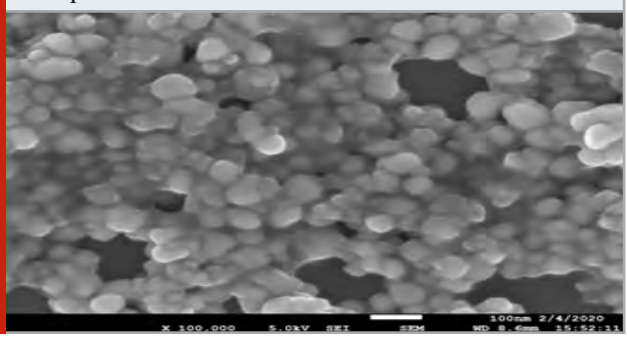
Figure 2: UV-vis absorption spectra of green synthesized silver nanoparticles at different incubation time.



pH analysis: When a *V. serpens* plant's extract was applied dropwise to the aqueous silver nitrate solution, immediately a color shift was observed, resulting in a pH reduction that can be a sign of silver nanoparticles synthesis. In this study the pH from high acidic to low acidic was observed.

FE –SEM analysis: For size distributions and morphological characterizations of synthesized silver nanoparticles, high-resolution FE-SEM images are used. FE-SEM study showed clearly that average size and shape of silver nanoparticles was 60-70 nm and spherical, respectively (Figure 3).

Figure 3: FE-SEM image of green synthesized silver nanoparticles.



FTIR analysis: FTIR experiment is used to describe surface chemistry of synthesiszed nanoparticle. FTIR finds organic functional groups such as OH, C=O, associated with the surface of nanoparticles (Pirtarighat et al., 2019). The solution that contains synthesized silver nanoparticles was centrifuged at 8,000 rpm for 10 min to isolate silver nanoparticles from other compounds and a deposit for FTIR analysis was prepared. The FTIRspectra of the green synthesized silver nanoparticles from V. serpens are shown in Figure 4 (Transmission) and Figure 5 (Absorbance). The FTIR peaks in the range of 1640 cm-1 that are applicable to C=O bond of the carbonyl group and the stretching vibrations of different amides also appeared in this range. The FTIR peaks in the range of 3200 to 3500 cm-1 were allocated as –OH stretching in phenolics and alcohol compounds with strong hydrogen bonds. Results showed the FTIR bands at 3894.7cm^{-1}, 3418.5 cm^{-1}, 2923.2cm^{-1}, 2566.4 cm^{-1}, 2321.0 cm^{-1}, 1622.6 cm-1, 1467.1 cm^{-1}, 1374.3 cm^{-1}, 1063.4 cm^{-1}, 946.5cm^{-1} and 537.4cm^{-1}. This demonstrated various functional groups linked to synthesized nanoparticles surface.

The existence of these peaks indicated that synthesized silver nanoparticles were covered by functional groups such as carboxylic acid, aldehyde, ketone, with plant secondary metabolites such as tannins, phenols, glycosides, flavonoids, terpenoids and others. The stability of the nanoparticles is responsible for the existence of these groups.

Figure 4: FTIR spectra of capped silver nanoparticles synthesized using *V. serpens* (Transmission).

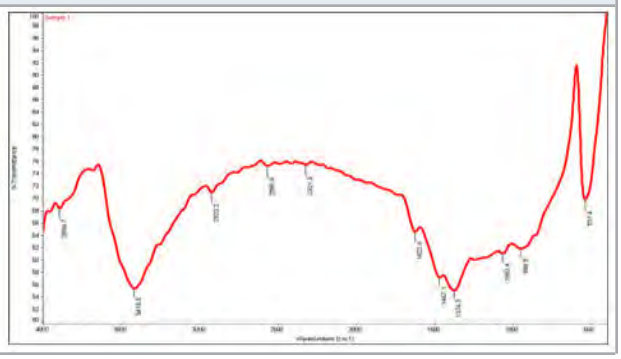
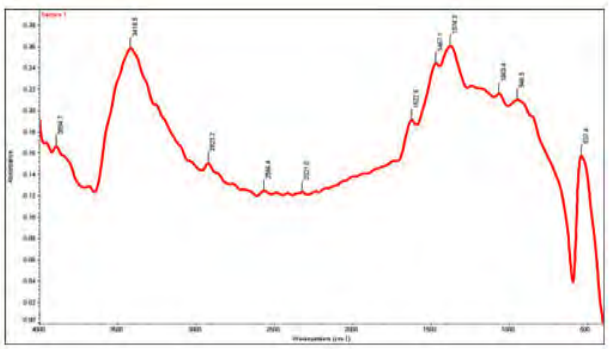


Figure 5: FTIR spectra of capped silver nanoparticles synthesized using *V. serpens* (Transmission).



XRD analysis: Figure 6 shows the XRD pattern gained for the green synthesized silver nanoparticles using the extract of *V. serpens*. The XRD analysis determined the crystalline nature, phase identification, and size of the silver nanoparticles. Comparison of the XRD spectrum with the Standard confirmed that our experiments made silver particles into nanocrystals, as evidenced by the 2θ peak value of 37.5873, 37.6909, 43.7941, 44.629, corresponding to (111), (200), (220) and (311) planes for silver, respectively. Unallocated peaks may be caused by the bio-organic crystallization on the nanoparticle surface.

Figure 6: XRD pattern of silver nanoparticles synthesized by using extract of *V. serpens*.

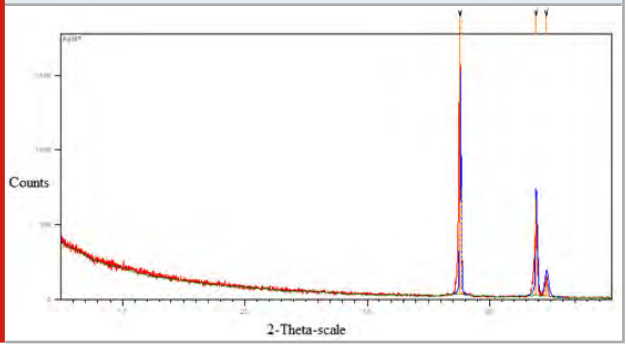
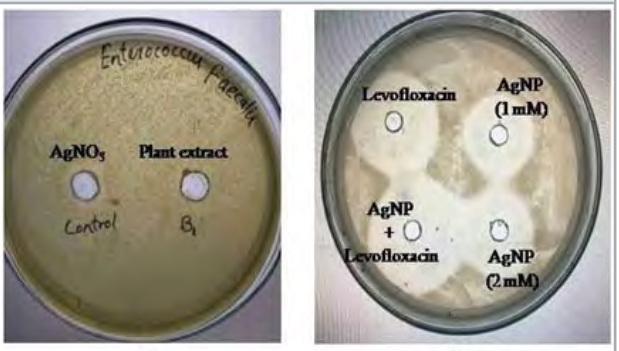


Figure 7: Antimicrobial Activity of (a) Plant extract and silver nitrate solution against *Enterococcus faecalis*; (b) AgNP (1 mM), AgNP (2 mM), standard antibiotic levofloxacin and synthesized AgNP with standard antibiotic levofloxacin against *Enterococcus faecalis*.



Antibacterial activity of silver nanoparticles: The results of our analysis have shown that *V. serpens* aqueous extract and silver nitrate solution showed no substantial inhibition of *Enterococcus faecalis* bacteria (Figure 7a) but the silver nanoparticles synthesized by green synthesis, inhibited the growth of *Enterococcus faecalis* (Figure 7b). In addition, synthesized silver nanoparticles showed a synergetic effect on the antimicrobial activity of the standard antibiotic levofloxacin against *Enterococcus faecalis* bacteria under this study (Figure 7b) (Table 1).

Table 1. Diameter of inhibition zone (mm)against *Enterococcus faecalis*.

Variables	Diameter of inhibition zone (mm)
Plant extract	10
$AgNO_3$	12
AgNP (1 mM)	18
AgNP (2 mM)	20
Levofloxacin (0.5 mM)	24
AgNP (1 mM) + Levofloxacin (0.5 mM)	28

CONCLUSIONS

The present study demonstrated an innovative way to use natural products to synthesize silver nanoparticles, which can be used in different biomedical applications. The synthesis of silver nanoparticles using *V. serpens* extracts as a green method without using any chemical stabilizer or reducer was reported. The findings from our study showed that the aqueous extract of *V. serpens* and silver nitrate solution did not show significant inhibiting activity against *Enterococcus faecalis* bacteria in tested concentrations but the green synthesized silver nanoparticles, inhibited the growth of *Enterococcus faecalis*. In addition, synthesized silver nanoparticles showed a synergetic effect on the antimicrobial activity of the standard antibiotic levofloxacin against Enterococcus faecalis bacteria under this study. Biosynthesized silver nanoparticles demonstrated a large range of antimicrobial susceptibility and thus are promising antimicrobial agents with potential biomedical uses.

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Conflicts of Interest: The authors declare no conflict of interest.

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Compassion Fatigue, Satisfaction and Burnout Among Oncology Nurses Working in Pediatric Oncology Setting

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ABSTRACT

A growing body of evidence suggests that burnout among oncology nurses is a significant result of the challenging and continuously high-stress work environment. It has been suggest-ed that professionals could be emotionally affected by young age of children receiving chem-otherapy, ethical decision making, observing the continuous suffering of children during or after chemotherapy. The main aim of this article is to examine the level of compassion fatigue, satisfaction and burnout experiences of oncology nurses when providing care to pediatric patients during chemotherapy. This article uses the qualitative descriptive (QD) research where it compares qualitative research with other types of qualitative methods. The article concluded that there is a severe shortage in Saudi nurses who works with pediatric oncology settings and highlights the importance to encourage Saudi nurses to work in specialized area with pediatric oncology. The achieved numerical statistical results have proven that success rates in treating patients at risk are low by about 85% and patients with moderate-risk conditions are about 7%. For patients with high-risk cases, the survival rate for a 3-year-old is 66%, and a 5-year-old is 60%. From the perspective of future actions, it is recommended to conduct in-depth research in brain tumors, retinal tumors, and kidney tumors that include Wilma tumor, neuroma, muscle cancer, soft muscle tissue tumors, germ cell tumors, liver tumors, and bone tumors including bone cancer, cancer Ewing, tissue disorders, such as histiocytosis of Langerhans cells and other rare tumors. It is highly preferred that patients be referred to hos-pitals before undergoing any major surgery or treatment, in order to present a comprehensive treatment plan to patients and their families, which provides the best opportunity for treat-ment..

KEY WORDS: ONCOLOGY, RADIATION THERAPY, PEDIATRIC, BURNOUT FATIGUE.

INTRODUCTION

Nurses work in shifts-usually 8 to 12 hours at a time and communicate with each other and exchange information to ensure that patient care is as smooth as possible when moving from one shift to another. They are usually responsible for caring for multiple patients during a single shift; For example, oncology nurses are responsible for caring for three patients at the same time. This applies most of the time to patients in the ICU due to their poor health and because they may be on special devices such as the ventilator, or receive medications that require constant monitoring and assessments. Also, the nurses lead the care process and take care of the daily needs of patients. Doubtless that caring for patients with cancer generates remarkable work-related stress that can result in nurses' dissatisfaction and mental exhaustion, (Ferrans, 1990).

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As compassion fatigue can influence patient satisfaction and safety, it is important for nurses to become knowledgeable about signs and symptoms of compassion fatigue as well as to be aware of strategies that can be utilized to develop a personal plan of care to avoid compassion fatigue. In the same vein,, a priority for healthcare systems is to provide healthy work environments for their staff and plan to address the needs of nurses who may be experiencing symptoms of compassion fatigue (Potter et al 2010, Lombardo & Eyre 2011).

On the other hand, patient and careers' satisfaction of care are crucial since high levels of sat-isfaction have been associated with higher quality of care and life (von Essen et al., 2002). Stamm (2010) defines professional quality of life as the quality one feels in relation to their work as a helper. Professional quality of life is the combination of both the positive quality (compassion satisfaction) and the negative quality (compassion fatigue). Additionally, In these clinical setting, nurses are expected to be proficient in the use of complex technologies and at the same time, engage in therapeutic communication with patients and families who have multiple needs that lead Pediatric oncology nurses to become overly involved with their patients and work to an extent of crossing professional boundaries (Zadeh, Gamba, Hudson & Wiener, 2012).

In pediatric oncology settings, studies of von Essen et al., (2001; 2002) suggest that compe-tence of the nurses and other healthcare professionals were pointed out as integral aspects of good quality care. Patient satisfaction is also influenced by the relationship between nurses and other healthcare practitioners and patients (Campbell et al., 2000). In a recent study con-ducted by Wangmo et al. (2016) in Switzerland, several aspects of care were identified as important in increasing the levels of satisfaction of both patients and their careers or family members. Results of this qualitative study suggest that helpful communication and respon-siveness or friendliness of the oncology nurses and other members of the healthcare staff were crucial in helping patients and their parents feel satisfied with the care they receive. Further, participants stated that when healthcare professionals go beyond the duty of care, their level of satisfaction with the care they receive is further increased.

Elbarazi et al (2017) stated that Saudi Arabia is among those Arab countries which despite its higher-income, is classified as a developing country based on international indicators. Elbarazi et al (2017) have reported the presence of a wide range of prevalence of high emotional exhaustion, high depersonalization, and low personal accomplishment. They concluded that the prevalence of burnout may be higher among health care professionals in Arab countries as their health systems and financing models are either weak, overburdened, or rapidly develop-ing and responding to the changing disease patterns and health status of the population. Many of these countries have a critical shortage of health care professional, particularly in some specialties such as (oncology Pediatrics settings), which may lead to overloading them with work responsibilities and making them prone to burnout. Compassion fatigue is often linked with burnout. The two have been identified as related but separate concepts (Yoder, 2010). Beck (2011) defines burnout as a psychological syndrome of emotional exhaustion, deperson-alization, and reduced personal accomplishment. Burnout develops gradually and has been identified as a subtle process during which a person is gradually caught in a state of mental fatigue and is drained of energy (Young, et al (2011); (Potter et al, 2010).

The second component of compassion fatigue, secondary traumatic stress, is a feeling of des-pair caused by the transfer of emotional distress from a victim to a caregiver that often devel-ops suddenly. In the presence of secondary traumatic stress, the caregiver is empathizing with the victim (Jahrami et al (2013). Although the elements of compassion fatigue are related, secondary traumatic stress is an effect of experiences with specific types of patients, whereas burnout is an effect of environmental stressors and is not unique to health care providers. The effects can be profound and potentially impact both the staff's personal quality of life as well as the work environment causing decreased productivity, a negative effect on the bottom line, difficulty recruiting, high turnover and increased sick days (White & Reg, 2006).

In the same vein, oncology nurses should provide support for patients and their parents in Pediatric oncology settings. Yoshida et al. (2014) observe that parents find it distressing to witness the suffering of their children, making decisions and preparations for palliative care and realizing that they could not do anything more for their children. The issue of emotional attachment has also been explored in the study of Dowling (2008), which suggests that nurse-patient relationship could help patients that they are better understood. However, the same relationship or intimacy might also impose emotional stress amongst nurses. Hence, appropri-ate understanding of the nature of nurse-patient relationship and/or intimacy is needed in or-der to minimize its negative effects on the nurses or patients. Nurses caring for Pediatric on-cology patients would also require support. It has been shown that nurses could feel isolation (Yoo et al., 2008). The ability of oncology nurses to explain technical terms in a simple manner was also singled out as important in increasing satisfaction (Wangmo et al., 2016).

The findings of Wangmo et al. (2016) are also similar to earlier studies (Von Essen et al., 2002; Campbell et al., 2000), which highlight how interpersonal factors are related to higher levels of satisfaction of care. A study that surveyed the level of satisfaction of young children or those who are 7–11 years old showed that caring healthcare providers, good communica-tion and trustworthiness were also factors that influenced the level of satisfaction of the chil-dren (Pelander, Leino-

Kilpi, & Katajisto, (2007). On the other hand, too little or too much information at the time of diagnosis when everything is difficult for members of the family and the patient to understand, could also lead to dissatisfaction (Clarke and Fletcher, 2003; Beck, 2014).

The most recent critical review of research evidence done by (Kirshbaum, 2020) is that phys-ical exercise and the treatment of underlying problems, such as anemia or clinical depression, are effective interventions. However, a wide range of practical interventions and complementary therapies are likely to be helpful such as: acupressure and acupuncture, stress management and relaxation, energy conservation measures, anticipatory guidance and preparatory information, and attention-restoring activities.

Significance of the present study: Working in the oncology, emergency and end of life settings can be full of stressful situations for patients, families and healthcare professionals. A growing body of evidence suggests that burnout among oncology nurses is a significant result of the challenging and continuously high-stress work environment. It has been suggested that professionals could be emotionally affected by young age of children receiving chemotherapy, ethical decision making, observing the continuous suffering of children during or after chemotherapy. Moreover, studies abroad have shown that a diagnosis of pediatric cancer causes emotional trauma and financial challenges to families and children. Additionally, Watt et al., (2013) have reported that the family-centred care model should be used when providing care in pediatric oncology settings.

Nurses who are involved in the care of pediatric patients with cancer also experience distress and in need of support in their role. However, there are still no studies conducted in Saudi Arabia and there is also a paucity of literature on the experiences of nurses of compassion fatigue, satisfaction, and burnout. Therefore, the researcher thinks about finding the prevalence of compassion satisfaction, compassion fatigue, and burnout among oncology nurses working in pediatric oncology setting since it affects the quality and safety of delivered care to patients and their families' satisfaction levels.

Conceptual Framework: Maslow's hierarchy of needs and Watson's theory of human caring were applied to guide studies related to compassion Fatigue (CF), burnout, and compassion Satisfaction (CS) (Burtson & Stichler, 2010). A most significant theoretical model developed by Figley (2002) who discovered that CF develops as a result of a caregiver's exposure to his or her patients' experiences joined with his or her natural empathy. Later on, Stamm (2010) applied theoretical path analysis diagram, a conceptual framework related to CS, CF, and burnout among nurses was developed to guide this study.

In the current study, the researcher believes that it's a cause and effect relationship between the individual and the organizational characteristics which may contribute to and have an influence on the development of CS, CF, and burnout. The demographic variables such as age, gender, level of education, years of experiences, hours of work per week, length of shift, are considered independent variables while the development of Compassionate fatigue and CF, and burnout will be the dependent variables in this study.

The main aim of this study was to examine the level of compassion fatigue, satisfaction and burnout experiences of oncology nurses when providing care to pediatric patients during chemotherapy. Specifically, this study aimed to address the following objectives: Describe the level of compassion fatigue and satisfaction of oncology nurses working with Pediatric patients. To Identify the manifestations of burnout experienced by nurse working in Pediatric oncology setting and assess the presence of secondary traumatic stress among nurses working in oncology Pediatrics settings. To explore the association between the nurses' personal and demographic characteristics and the level of compassion fatigues, satisfaction and burnout. There are three types of BCI systems: invasive, semi-invasive, and non-invasive Gonfalonieri (2018).

METHODOLOGY

Design: across sectional survey was used to achieve the purpose of this study. Study subjects: All registered nurses (RNs) who worked in inpatient and outpatient's clinic of oncology Pediatrics setting at three hospitals in Jeddah. The inclusion criteria for participation was (a) work at least 8 hr per week in the oncology Pediatrics setting, (b) interact directly with Pediatrics cancer patients and their families at least 8 hr per week, and (c) have at least 1 year of experience in the Pediatric oncology setting. This inclusion criteria was applied to make sure that the recruited nurses were spent enough to traumatic events that contribute to the development of CF and burnout. Minimum of 150 nurses were expected to participate by using a convenient sampling technique.

Data Collection Tools: The tool of the current study consisted of 2 parts as following: 1.A demographic questionnaire which was developed by the researcher and enquire about age, educational level, marital status, years in nursing profession, typical shift length, years of experiences in oncology setting. etc. 2.The Pro QOL scale, version 5, this scale used in this study based on many pervious researches who was used to examine the prevalence of CS, CF, and burnout among nurses Permission to use the Pro QOL instrument was granted via the website of the tool's author. The Pro QOL tool was first developed in 1995 and has been used, revised, and updated over time. The scale is consisting of 30 items self-report survey that includes three subscales: CS, CF, and burnout (Figley & Stamm, 1996). Testing for convergent and discriminant validity have demonstrated that each scale measures different constructs (Stamm, 2010).

Each subscale is distinct, and the results of each subscale cannot be combined to give single significant score. The Pro QOL scale consists of 3 subscales (compassion satisfaction, burnout, and secondary traumatic stress) used to measure compassion satisfaction and compassion fatigue. Burnout and secondary traumatic stress are components of compassion fatigue, whereas compassion satisfaction is a stand-alone measure. Each subscale has 10 question items and uses a 5-point Likert scale scoring from 1 = never to 5 = very often (Stamm, 2010). The scores of the ProQOL for each subscale were totaled using Stamm's validated levels: a CS score of 22 or less denotes low levels of CS, a score of 23–41 indicates average levels, and 42 and above suggests high levels of CS. For CF and burnout, a score of 22 or less indicates low levels, 23–41 indicates average levels, and a score of 42 and higher reveals high levels of CF.

Validity And Reliability: The Pro QOL scale was translated into Arabic and back translated into English, verifying whether the translation covers all aspects of the original English version of the questionnaire or not. To ensure the face validity of the final translated Arabic version of the questionnaire it was evaluated by experts who selected based on their qualifications and experience in nursing research and education. Then, the tool was piloted and tested by 10 participants to identify ambiguities, the time required and any difficulties that might be encountered by the participants in reading or understanding. The reliability of the questionnaires was calculated, and Cronbach Alpha for CF, CS and burnout questionnaires will be reported later.

Data Collection and Management: The data were collected primarily through questionnaires specifically developed to address the research objectives. A formal invitation letter was sent through mail or direct contact to qualify registered nurses. The mail contained detailed study information, objectives, data collection process, why they are chosen as participants, the significance of the information sought, and the confidentiality of their information. Once they agreed to participate in the study, a written informed consent was obtained, and participant was assigned an identification code. The scale along with demographic personal data sheet were distributed to the participants and enough time was given to fill the questionnaire since they were busy and overloaded by their work. Data collection for this study were run for a period of maximum 2 -4 weeks.

When the subjects returned the questionnaires, the researcher was reviewing its veracity and completeness. In cases of incomplete or incorrectly filled questionnaires, the researcher was had the option to call the participant for clarifications or consider as missed data. Data were analyzed by using the Statistical Package for the Social Science (SPSS) for Windows, version 22.0 (SPSS Inc.). Item means, standard deviations, medians, and percentages of the descriptive statistics was computed for the level of CS, CF, and burnout. A series of Pearson r correlation, t test, and one-way analysis of variance (ANOVA) used to examine the associations between demographics, work-related characteristics, and the level of CS, CF, and burnout.

The statistically significant level calculated at .05. Multiple regression employed to determine which variables of demographics and work-related characteristics contributed to the variation of the level of CS, CF, and burnout among subjects. Using seven selected independent variables to run a multiple regression, this study needs a minimum sample size of 153 subjects to achieve 95% power and a medium effect size (.15) at α = .05.

Demographic and personal information: Table 1 shows that the higher percentages of pediatric oncology nurses age group were found with the young ages and the percentage decreases when the age increase.

Table 1. Age group for the participating parents

Age of the parents	%
21-30 years	34 %
31-40 years	39 %
41-50 years	23 %
51-60 years	4%

Figure 1: Age group for the participating parents

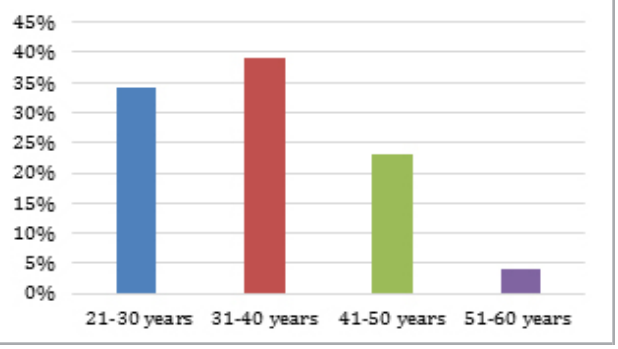


Table 2 shows the experiences years of nurses who work in pediatric oncology which decreases to 3 % when it is more than 20 years.

Table 2. Years of pediatric oncology nursing experiences

pediatric oncology nursing experiences	%
Less than 5 years	32 %
5-10 years	41%
10-15 years	17%
16-20 years	7%
More than 20 years	3 %
Total	100 %

Figure 2:Years of pediatric oncology nursing experiences

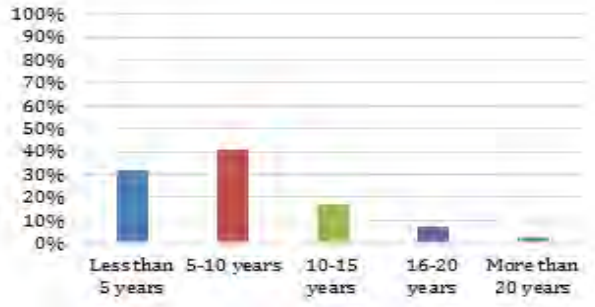


Table 3 shows that there was a severe shortage in Saudi nurses who work with pediatric oncology settings and highlights the importance to encourage Saudi nurses to work in specialized area with pediatric oncology.

Table 3. Nationality

Nationality	%
Saudi	9 %
None Saudi	91 %
Total	100%

Figure 3: Nationality

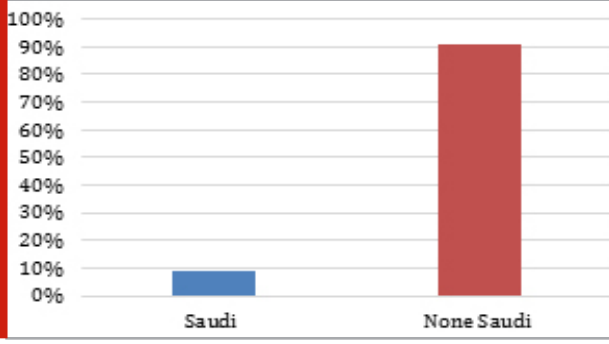


Table 4 shows there is low number of male nurses who works with pediatric oncology settings.

Table 4. Gender

Gender	%
Male	12 %
Female	88 %
Total	100 %

Table 5 shows that most the oncology nurses who works with pediatric settings have diploma level of education.

Table 6 shows the Professional quality of life scale (PROQOL) which shows average Level of Compassion (54 %), High Level of Burnout (60 %) and Average Level of Secondary Trauma (54 %).

Figure 4: Gender

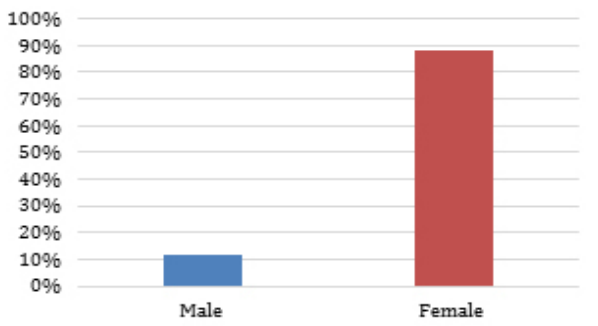


Table 5. Edcuation

Level of education	%
Diploma level	79 %
College level	21 %
Total	100%

Figure 5: Education

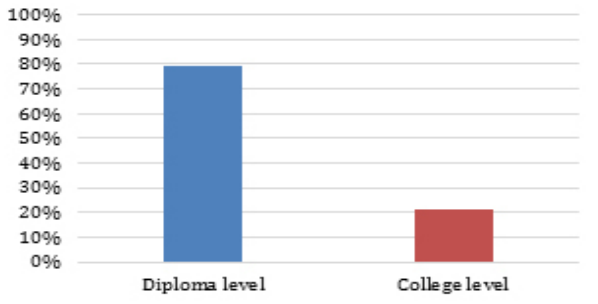
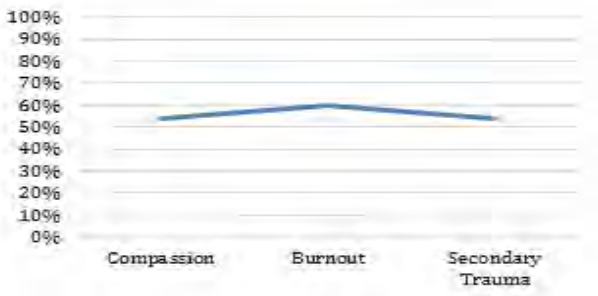


Table 6. Professional quality of life scale (PROQOL)

Level	Score
Lower than average	43 or less
Average	Around 50
High	57 or more

Figure 6: Professional quality of life



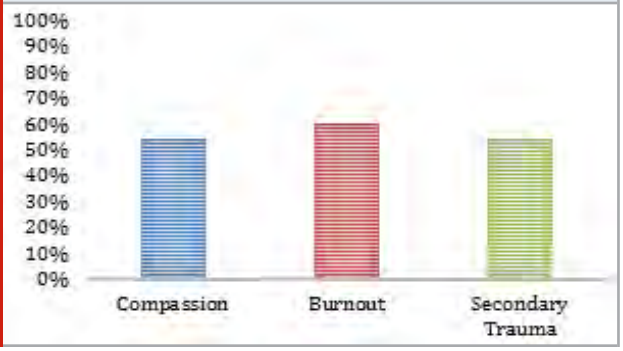
RESULTS AND DISCUSSION

group were found with the young ages and the percentage decrease when the age increase, this is due that most of young ages are newly graduate and newly work with pediatric oncology and didn't experience Burnout or Secondary Trauma while the lower number of old ages present the nurses who manages to cope with the nature in working with pediatric oncology settings. It also reflects on the experiences years of nurses who work in pediatric oncology which decreases to 3 % when it is more than 20 years. There is a severe shortage in Saudi nurses who works with pediatric oncology settings and highlights the importance to encourage Saudi nurses to work in specialized area with pediatric oncology.

Table 7. Professional quality of life

Professional quality of life	Level	%
Compassion Satisfaction status	Average Level of Compassion	54 %
Burnout status	High Level of Burnout	60%
Secondary Trauma status	Average Level of Secondary Trauma	54%

Figure 7: Professional quality of life



There is low number of male nurses who works with pediatric oncology settings, this require encouraging male in Saudi Arabia to join nursing as a profession in Saudi Arabia and encourage to work in specialized area like pediatric oncology stings. Most the oncology nurses who works with pediatric settings have diploma level of education, which indicate the sever need to provided programs for bachelor's degree in nursing for female and male nurses in Saudi Arabia. The Professional quality of life scale (PROQOL) which shows average Level of Compassion (54 %) which indicate the most of the nurses in the sample were showing satisfaction with their profession, High Level of Burnout (60 %) which was noted due to their work with pediatric oncology patient, Average Level of Secondary Trauma (54 %) which indicated the risk to develop Secondary Trauma due to their work with pediatric oncology patients and to deal with these patients for long time.

We reached to the main finding that assure to reduce fatigue severity and distress and its impact on functioning, intensified collaborations and close partnerships between clinicians and researchers are needed, with an emphasis on system-wide efforts to disseminate and implement these evidence-based recommendations. The scale of fatigue in children with cancer is a reliable and valid instrument to measure the level of fatigue. The scales are brief, reliable, and feasible to assess multi-dimensional aspects of fatigue among such children. The instrument helps health professionals to monitor fatigue in children with cancer, (Mahdizadeh et al, 2020).

CONCLUSION

Nurses who are involved in the care of Pediatric patients with cancer also experience distress and in need of support in their role. However, there are still no studies conducted in Saudi Arabia and there is also a paucity of literature on the experiences of nurses of compassion fatigue, satisfaction and burnout. Therefore, the research thinks about finding the prevalence of compassion satisfaction, compassion fatigue, and burnout among oncology nurses working in Pediatrics oncology setting since it affects the quality and safety of delivered care to patients and their families' satisfaction levels. This study clarifies the light on finding the level of compassion fatigue, satisfactions and burnout among nurses working at Pediatric oncology setting since these increased levels of burnout affect not only the quality of nursing interventions provided to the patients but also it has a greater impact on the oncology nurses' stress-related symptoms, job dissatisfaction, decreased productivity, safety issues, and job turnover within the healthcare system.

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Perception of Smile Esthetics -A Questionnaire Based Study

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ABSTRACT

Smile is the most indicating feature of a individual's physical and social well-being. Most of the cosmetic treatment are aimed at achieving an ideal smile through improving various features of the smile. The predominant notable characteristic of the smile includes the dental hard and soft tissues which are at fault for many and subsequent orthodontic opinion are sought. From an orthodontist point of view, successful orthodontic treatment concerns about finishing the case according to the certain guidelines which in general includes dental relationships like dentition in occlusion, functional occlusion and parallel roots on panoramic radiograph with an esthetic soft tissue profile. But on the contrary, the patient views only the esthetic soft tissue profile and few visible dental features upon smiling. This study aimed at evaluating the perception of various features of smile esthetic by patients visiting orthodontic fraternity. The samples for the study were chosen among patients who visited Saveetha dental college for their orthodontic consultation. From among the orthodontic outpatients, 60 respondents (30 male and 30 females) between the age group of 15-30 years were randomly chosen.

Patient's consent to participate in the study was initially obtained. Few esthetically appealing posed smiles photographs with almost ideal occlusion were chosen. Using a photo modifying software, the photographs were then altered and arranged in a cluttered sequence where each set represented variations in particular dental and smile feature. Visual Analog scale was used to score these photographs. This consisted of a scale of values from 1 to 100 with labelling of least attractive towards the left which was coded as zero to right extreme at 100 mm which was labelled as very attractive. Data was tabulated and descriptive and inferential (independent t test) statistics were calculated. No Gingival show more than 1-2 mm, increase of 1 mm above the average crown exposure, presence of midline diastema was considered to be less esthetic Difference in the perception between the male and female population($p>0.05$) was noted to be very minimal in the present study indicating an equally increasing awareness in both the genders. With increasing awareness among the laypersons, the decision regarding the treatment can be made a combined decision between the orthodontist and the layperson.

KEY WORDS: SMILE ESTHETICS, PATIENT PERSPECTIVE, PHOTOGRAPHIC SMILE ASSESSMENT, DENTAL ESTHETICS, VISUAL ANALOG SCALE.

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INTRODUCTION

Smile is the most indicating feature of an individual's physical and social well-being. It exhibits attractiveness to a patient appearance and also improves the self-confidence of an individual in the society. Most of the cosmetic treatment are aimed at achieving an ideal smile through improving various features of the smile. The predominant notable characteristic of the smile includes the dental hard and soft tissues, which are at fault for many for which subsequent orthodontic opinion are sought. Majority of the patients seek orthodontic treatment with a concern to improve smile esthetics (Shaw et al., 1985). Soft tissue drape either exhibits the problem of the underlying teeth and bone or it masks the problem by compensation. Achieving an esthetic smile is based on our understanding about various components of the smile and the balance that exists between hard and soft tissues.

Every coin has two sides, as with the saying, any orthodontic treatment need raised by the patient has two perspectives. Successful orthodontic treatment, in the orthodontist point of view, it concerns about finishing the case according to the certain guidelines which in general includes dental relationships like dentition in occlusion, functional occlusion and parallel roots on panoramic radiograph with an esthetic soft tissue profile. But on the contrary, the patient views only the esthetic soft tissue profile and few visible dental features upon smiling. Studies have shown a poor correlation between the patient perception and dentist consideration of these guidelines (Almanea et al., 2019). Difference in opinion exists even among the specialist pertaining various dental specialties. The orthodontist, periodontist usually be the critical reviewers followed by the endodontist and the prosthodontist compared to any other dental speciality. Hence apart from obtaining a good occlusion, consideration of the soft tissue in treatment planning holds utmost importance for an esthetic outcome (Khalil, 2019, Pinzan-Vercelino et al., 2020).

Hence soft tissue components should also be considered in these to improve the attractiveness of the smile (Schabel et al., 2008). The esthetic consideration of smile varies among people from different national and cultural background. Deviation from normal value can be acceptable in different population while achieving esthetic smile for a patient orthodontically (Jayachandar and Dinesh, 2016; Kamath and Arun, 2017; McLeod et al., 2011; Sridharan and Samantha, 2016). Several parameters of the facial features are considered to rate the attractiveness of the smile and these parameters vary among both the genders (Godinho et al., 2020).

Smile esthetics seems to be affected by a variety of dental features, each with a varied degree of threshold including the lateral negative space, arch with, shape, teeth shape, any other dental asymmetries and also the age of the population examined. Among the various factors, the presence of anomalies in the shape of the teeth seemed mostly to affect smile esthetics (Kau et al., 2020). Standard orthodontic records include the photographs, radiographs and dental cast. They serve as an ideal replica of the hard and soft tissues. Photograph gives us a wide knowledge about the patient's smile and the soft tissue facial features(Havens et al., 2010; Howells and Shaw, 1985; Schabel et al., 2010).

Extraoral facial photographs depict the patient features at rest and during smile. Of the various possible smile, posed smile is one where voluntary smile is elicited and it is not driven by any emotions. It is usually a learned greeting and can be sustained and is reproducible. This is used in evaluation for orthodontic purposes (Ackerman et al., 1998). The smile features like the amount of lateral negative space visible, smile arc, smile line and symmetry of the smile are among the most importantly considered factors by many orthodontist and these factors altogether can be diagnosed from a frontal and oblique view photographs which hence form a essential modality for diagnosing orthodontic cases (Kadhim et al., 2020).

Variation in type of extraction plan has no effect on the smile esthetics. But rather it depends on the way in which the treatment plan is executed. Hence proper planning of various stages of treatment is more essential for success of orthodontic treatment (Janson et al., 2011). But in a study by Kim et al this concept has been disproved since no difference in change in arch width is noted among the two treatment options (Kim and Gianelly, 2003). Also type of the fixed appliance used doesn't have any difference in the smile esthetics, only the mechanics plays a role (Negreiros et al., 2020). Whereas another study had concluded that different modalities of the treatment have varied effect on the smile features when concerned like in patients treated with fixed appliance therapy, the amount of lateral negative space was reduced with increased dental show when compared to the patients treated with functional appliance therapy (Shoukat et al., 2020).

Previous literature evidences rule out the void between the patient desires and an orthodontist opinion on ideal occlusion. With a shift towards soft tissue paradigm, it is essential to consider the patient opinion regarding the final outcome of the treatment when deciding the treatment plan and the mechanics with which it would be achieved. This study aimed at evaluating the perception of various features of smile esthetics by the patients(non-dentist) visiting orthodontic fraternity (Shoukat et al., 2020).

Objectives: The objectives of the study were to evaluate patient perspective in visualizing the various features in a posed smile, especially, Level of Visibility of Gingival Margin, Crown Height of Central Incisors, Lateral Negative Space, Midline Diastema- Upper Central Incisors, Midline Deviation- Lower, Crown Angulation of
Central Incisor

MATERIAL AND METHODS

Study Sample: This was a cross sectional study done among patients who visited Saveetha dental college for their orthodontic consultation. From among these patients, 30 male and 30 female participants were randomly chosen. All these participants were in the age group of 15-30 years and had internal motivation for need for orthodontic treatment. Followed by explanation of the purpose of the study, with the respondent's consent, questionnaire was distributed.

Questionnaire: This consisted of 7 different smile photographs each one was used to depict a particular dental feature. Only ideal smile component was chosen and using a digital photo modifying software, each photograph was then altered (Adobe Systems Inc.) with few variations in each of the dental features specified. The alterations in these photos were made such that they quantify an increasing order of the particular discrepancy. Seven sections of the photographs were included and under each of these the photograph depicting a particular parameter was jumbled and arranged such that they do not follow a constantly increasing pattern.

Several methods are available to measure individual perceptions of a particular feature(Phillips et al., 1992). Few of which includes the Q sort analysis and use of grading scales. Grading scales provide us a knowledge about exacting responses in comparison. Each of these methods has advantages and limitations (Schabel et al., 2009). Grading Scales are used for a variety of purpose including the perception of pain (Almoammar et al., 2020), effect of certain medicaments, anxiety (Gazal et al., 2016), assessment of esthetics (Cosyn et al., 2017). The major advantage with the use of such calibration was the continuum scale in which the number of features can be rated and the arithmetic mean and standard deviation would provide needed details regarding the discrete features (Fowler et al., 2019).

Table 1. Smile Parameters Mentioned in survey

Parameter	Increments of change
Level of visibility of gingival margin	Increased in an increment of 0.5 mm from 0.5mm to 2.5 mm (Figure 1)
Crown Height of Central incisors	Increased in an increment of 0.5 mm from less than 0.5mm to 2.5mm (Figure 2)
Lateral negative space	Increased in an increment of 1 mm from 0-5 mm. (Figure 3)
Midline diastema- Upper central incisors	Increased in an increment of 0.5 mm from 0mm- 2.5 mm (Figure 4)
Midline deviation- Upper	Increased in an increment of 0.5 mm from 0mm- 2 mm (Figure 5)
Midline deviation- Lower	Increased in an increment of 1 mm from 1-5 mm. (Figure 6)
Crown angulation of central incisor	Increased in increment of 5 degrees. from 0-15 degrees. (Figure 7)

Figure 1: Level of visibility of gingival margin- Increased in an increment of 0.5 mm from 0.5mm to 2.5 mm

Figure 2: Crown Height of Central incisors- Increased in an increment of 0.5 mm from less than 0.5mm to 2.5mm

In the present study, VAS (Visual analog scale) was used to rate these photographs. This consisted of a scale of values from 1 to 100 with labelling of least attractive toward the left corner which was coded as zero to right corner at 100 mm which was labelled as very attractive. Hence the participants after visualizing the set of pictures under each subheading, marked a score of 1-100 for each of the photograph. Pre testing of the questionnaire was initially was done by the examiners among few out patients and the ability to process the details in

the photographs were analysed. After modifications, questionnaire consisting of seven dental features where shown to the patient ratings were graded based on the patient evaluation.

Statistical Analysis: Scores for each of the modified photograph along with the original photograph were then entered in an excel sheet and the results were summarized. Data were then transferred to a SPSS (version 26.0) software and statistical analysis were performed. Independent samples t test was done between the male and the female population regarding their opinion about the individual smile parameters.

Figure 3: Lateral negative space - Increased in an increment of 1 mm from 0-5 mm.

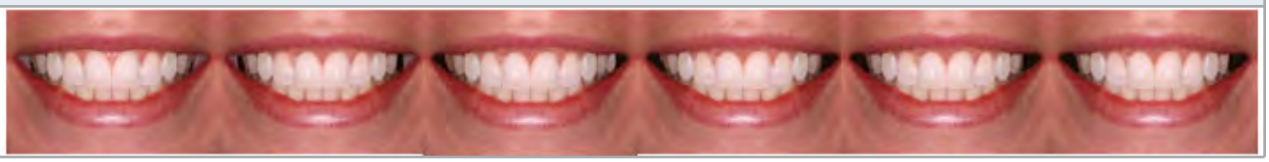


Figure 4: Midline diastema- Upper central incisors- Increased in an increment of 0.5 mm from 0mm- 2.5 mm

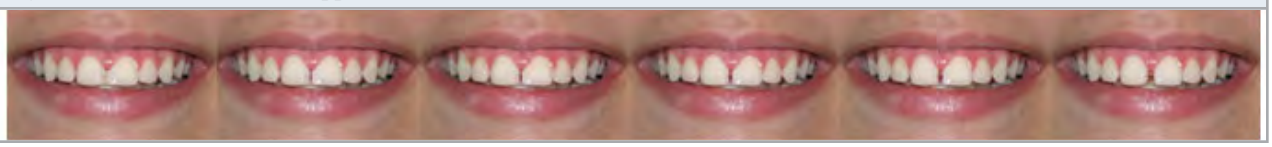


Figure 5: Midline deviation- upper- Increased in an increment of 0.5 mm from 0mm- 2 mm

Figure 6: Midline deviation- Lower- Increased in an increment of 1 mm from 1-5 mm

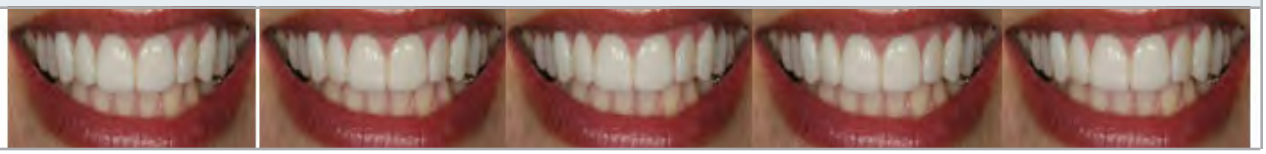
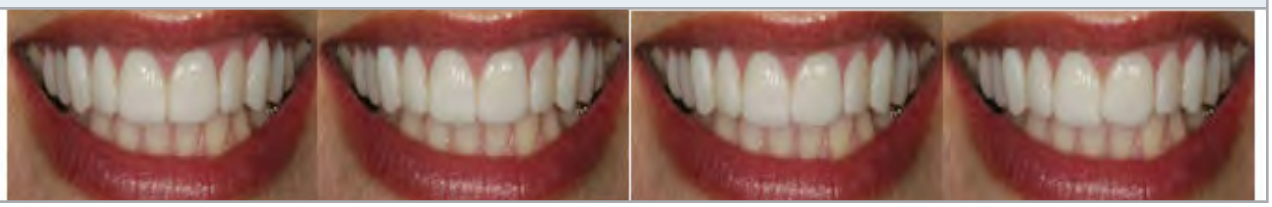


Figure 7: Crown angulation of central incisor- Increased in increment of 5 degrees from 0-15 degrees.



RESULTS AND DISCUSSION

The purpose of this study was to evaluate the patients' perception of the various smile features that is considered in any routine orthodontic diagnosis and treatment planning. The patient expectation and the orthodontist perspectives are two sides, the harmony between which brings an excellence in outcome. Orthodontist desires to bring an occlusal and functional balance wherein patients' considerations either deal with esthetics or function. Considering a patient with esthetic demands, it becomes essential to visualize the patient expectation. With a shift in trend towards the soft tissue paradigm and analysis of micro and mini esthetic features helps in better understanding of an individuals' perception of these features and thereby help us evaluating and planning accordingly (Sarver, 2015).

Features of the smile considered in the study were gingival show in the anterior region, crown height of centrals with respect to the adjacent laterals, lateral negative space, midline discrepancies in upper and lower arch, midline diastema, Crown angulation of the centrals with respect to the adjacent central incisors. Totally 60

patients in the age group of 15-30 years participated in the study of which 30 patients were male and 30 were female. Overall, the results of the study showed no statistically significant differences in the perception between the male and female study population for the parameters considered.

Gingival Show: In the present study increase in gingival show of about 1-2 mm was considered to be more esthetic by most by both the male and female population whereas increased show more than that was considered to be less esthetic. In a similar study by Talic et al in Saudi population it is shown that people there had lesser threshold for excessive gingival show. High gingival smile line was said to be unpleasant. But this concept is recently being disproved(Peck and peck, 1992; Talic et al., 2013) Age had an effect on the amount of gingival show in anterior region. With age the gingival show comparatively decreases and this was one of the pleasing characteristics according to elderly people.

Table 2. Smile Parameters Gingival exposure, Crown height of centrals, Lateral negative space.

FEATURE		GENDER	MEAN	STD. DEVIATION	P VALUE
GINGIVAL EXPOSURE	GM 0	MALE	59.5000	7.11361	0.588*
		FEMALE	58.50	7.089	
	GM 1	MALE	64.5000	7.11361	0.588*
		FEMALE	63.50	7.089	
	GM 1.5	MALE	69.5000	7.11361	0.588*
		FEMALE	68.50	7.089	
	GM 2	MALE	54.5000	7.11361	0.588*
		FEMALE	53.50	7.089	
	GM 2.5	MALE	54.5000	7.11361	0.588*
		FEMALE	53.50	7.089	
CROWN HEIGHT OF CENTRALS	CH 0	MALE	69.5000	7.11361	0.588*
		FEMALE	68.5000	7.08933	
	CH 0.5	MALE	69.5000	7.11361	0.588*
		FEMALE	68.5000	7.08933	
	CH 1	MALE	69.5000	7.11361	0.588*
		FEMALE	68.5000	7.08933	
	CH 1.5 MALE	64.5000	7.11361	0.588*	
		FEMALE	63.5000	7.08933	
	CH 2	MALE	69.5000	7.11361	0.588*
		FEMALE	58.5000	7.08933	
LATERAL NEGATIVE SPACE	LNS 0	MALE	69.5000	7.11361	0.588*
		FEMALE	68.5000	7.08933	
	LNS 1	MALE	69.5000	7.11361	0.588*
		FEMALE	68.5000	7.08933	
	LNS 2	MALE	64.5000	7.11361	0.588*
		FEMALE	63.5000	7.08933	
	LNS 3	MALE	58.5000	8.21584	0.871*
		FEMALE	58.1667	7.59802	
	LNS 4	MALE	53.5000	8.21584	0.631*
		FEMALE	54.5000	7.80694	
	LNS 5	MALE	53.5000	8.21584	0.631*
		FEMALE	54.5000	7.80694	

(*P value> 0.05 hence statistically not significant)

On the contrary, young individuals consider visibility of the incisor upto a certain length to be more esthetic. (Sriphadungporn and Chamnannidiadha, 2017). Also in a recent study, ratter's in all age group have identified reduced attractiveness of the smile with increased gingival show among both the gender (Tosun and Kaya, 2020). Reduction in the amount of upper incisor display and increased optimal display of the lower incisor were

believed to be more esthetic. In such a situation ensuring patient satisfaction becomes our utmost priority, treating the patients based on their likes and dislikes. (Table 2).

Crown Height of Centrals: Crown height measured from the incisal edge to the gingival margin was altered in increments of 0.5 mm. In the present study from the values obtained, ability to identify the differences in the crown height was competitively low among the laypersons. In orthodontics, during treatment planning the average height differences considered in between the gingival levels of central and lateral was found to be 1.23mm, central being a at a higher position than the laterals (Hourfar et al., 2019).

Table 3: Smile Parameters Midline discrepancy (Upper and lower), Midline diastema, Crown Angulation

FEATURE		GENDER	MEAN	STD. DEVIATION	P VALUE
MIDLINE DISCREPANCY- UPPER	MD 0	MALE	88.5000	7.32850	0.858*
		FEMALE	89.5000	6.99137	
	MD 0.5	MALE	77.8333	8.06048	0.870*
		FEMALE	78.1667	7.59802	
	MD 1	MALE	58.5000	8.21584	0.871*
		FEMALE	58.1667	7.59802	
	MD 1.5	MALE	53.1667	10.94527	0.601*
		FEMALE	54.6667	11.13656	
	MD 2	MALE	45.1667	13.42176	0.277*
		FEMALE	41.1667	14.77902	
MIDLINE DISCREPANCY- LOWER	MD 0	MALE	87.3333	7.39680	0.858*
		FEMALE	87.6667	6.91492	
	MD 1	MALE	77.8333	8.06048	0.870*
		FEMALE	78.1667	7.59802	
	MD 2	MALE	58.5000	8.21584	0.871*
		FEMALE	58.1667	7.59802	
	MD 3	MALE	44.3333	14.66484	0.931*
		FEMALE	44.0000	15.10880	
	MD 4	MALE	37.6667	13.87961	0.390*
		FEMALE	34.5000	14.46458	
MIDLINE DIASTEMA	0MM	MALE	69.5000	7.11361	0.870*
		FEMALE	87.6667	6.91492	
	0.5MM	MALE	69.5000	7.11361	0.870*
		FEMALE	83.1667	7.59802	
	1 MM	MALE	64.5000	7.11361	0.871*
		FEMALE	78.1667	7.59802	
	1.5 MM	MALE	58.5000	8.21584	0.871*
		FEMALE	58.1667	7.59802	
	2MM	MALE	53.5000	8.21584	0.631*
		FEMALE	58.1667	7.59802	
	2.5MM	MALE	53.5000	8.21584	0.897*
		FEMALE	54.5000	7.80694	
CROWN ANGLATION	0 DEG	MALE	92.6667	5.04007	0.198*
		FEMALE	92.8333	4.85715	
	5 DEG	MALE	64.3333	6.53021	0.770*
		FEMALE	61.8333	8.25074	
	10 DEG	MALE	28.5000	6.03867	0.831*
		FEMALE	29.0000	7.11967	
	15 DEG	MALE	12.8333	6.25373	0.591*
		FEMALE	13.1667	5.79586	

(*P value> 0.05 hence statistically not significant)

The discrepancies in the gingival show between the central and lateral incisor was a better tolerable feature by the lay person and most of them fail to appreciate this feature. The level of tolerance for this was noted to be 2mm (Ker et al., 2008). The labiolingual position of the incisors seem to be more evident when viewed by dentist or the layperson where dentist scored much lesser compared to the others(Jiang et al., 2020). Crown height measured from the incisal edge to the gingival margin was increased in increments of 0.5 mm. Increase in about 1 mm of the average crown exposure was considered to be normal. Increase in the height more than 1 mm is considered to be less esthetic.

Increase of more than 2 mm is considered to be the least attractive feature when smile is visualized which is similar to the results of the study by Talic et al where in Saudi population the layman perceived smile to be un-esthetic when it exceeds 2 mm than the average values (Talic et al., 2013).Also in a recent study, it had been evident that among the orthodontic patients, the width and height ratios were highly variable and doesn't correlate with the provided ideal values and the population are mostly unaware of such deviations that it doesn't make it a necessity for them to orthodontically correct such dental variations (Iftikhar and Roghani, 2020) (Table 2).

Lateral Negative Space: Lateral negative space is measured as the distance between the most posterior visible tooth to the corner of the lips during smile. Increase in the lateral negative space decreases the attractiveness of the smile for the patient. In an ideal smile the right and left side corridor spaces are equal. In men the negative space was comparatively larger than those in the female (Ritter et al., 2006). Moore et al described laypersons perception of an ideal smile was broader smile with ideal buccal corridor space (Moore et al., 2005; Ritter et al., 2006) . Descending perception of attractiveness was noted in the present study with increasing lateral negative space from 1 to 5 mm, this is in accordance to the study by Parekh et al (Parekh et al., 2006).

On the contrary in a study by Sabrina et al buccal corridor space was said to have a very little or almost no effect in the facial attractiveness of the patient (Zange et al., 2011). Gender differences in the amount of lateral negative space is present hence treatment formulation also varies between male and female population (Hadi et al., 2020). Also in a study among North Indian subjects, the more number of pleasing smiles were found in association of decreased buccal corridor space (Janu et al., 2020). Both dentist and the layperson perceive increase in the buccal corridor as an unaesthetic characteristic and such patients with increased lateral negative space were more ideal for undergoing orthodontic treatment on esthetic point (Golshah et al., 2020; Ioi et al., 2009, 2012; Ks et al., 2020). Also the lateral negative space was the most variable factors even in people with esthetically appealing smiles (Chen et al., 2020) (Table 2).

Midline Diastema: The presence of midline diastema was considered to be less esthetic than one without it. In patients increase in diastema of more than 2 mm was considered to be least esthetic than any feature deviations. The level of tolerance to midline diastema unlike any other dental anomaly was greatly increased. This was in accordance with few previous studies where in different population the appearance of the space between tooth was considered to be least tolerant un-aesthetic feature.(Bolas-Colvee et al., 2018) Diastema was a notable dental deformity and orthodontist seem to rate it better compared to laypersons(Tanaka et al., 2020) (Table 3).

Dental Midline Discrepancy: Minor deviations usually from the normal occurs in almost all the individuals. But in case of deviations more than certain acceptable levels are perceived to be un-aesthetic. Perception of esthetics varies among almost all individuals. Perception of the dentists have been studied so far concluding that dentists perceive even the minor deviation from the normal values. But decision regarding need for treatment is always in the patient view regarding every aspect of the malocclusion. The perception of the non-dentist regarding the upper dental midline shift showed that the upper dental midline shift was better perceived by the layperson. A deviation of more than 2mm was considered to be un-aesthetic. This is similar to a study by Talic et al among the Saudi population and Kokich et al among the American population where similar perception among laypersons were noted. The order of discrepancy of the midline is as follows, mandibular dental midline deviation was seen in 62% of patients, followed, in descending order of frequency, by lack of dental midline coincidence (46%), maxillary midline deviation from the facial midline (39%), molar classification asymmetry (22%), maxillary occlusal asymmetry (20%), mandibular occlusal asymmetry (18%), facial asymmetry (6%), chin deviation (4%), and nose deviation(3%)(Sheats et al., 1998).

Also, a threshold difference had been noted among the dentist and lay person regarding the perception of the upper dental midline deviation. Dentist in the previous studies have reported that they consider even 1mm of the midline deviation in the upper arch to be less esthetic. Even in those celebrities, who were said to have pleasing smile, persistence of the midline deviation was higher compared to any other dental asymmetry(Arroyo Cruz et al., 2020). This is probably because of the professional expertise of the dentist when compared to the patients in making out the minor deviations which in general are left unnoticed unless there is a notable deviation in the midlines. (Table 3).

Crown Anglation: Regarding the change in the inclination of the maxillary incisor tooth, 10 degrees and more than that from the normal inclination was rated to be comparatively unaesthetic. This is not in accordance to the similar study among the laypersons and dentists where only more than 15 degrees was considered to be unaesthetic by the Saudi population.(Talic et al., 2013)

This might be because of the increasing awareness among patients and their ability to judge even the minor disturbances in the dental esthetics. Also, these values are very similar to the dentist perception of the midline deviation where the even minor deviation in the axial inclination of the tooth was considered to be less esthetic(Williams et al., 2014).

CONCLUSION

Within the limitations of the study, it can be concluded that attractiveness of face is inversely related to the presence of lateral negative space. Gingival show more than 1-2 mm, increase of 1 mm above the average crown exposure, presence of midline diastema was considered to be less esthetic Difference in the perception between the male and female population was noted to be very minimal in the present study indicating an equally increasing awareness in both the genders. With increasing awareness among the laypersons, the decision regarding the treatment can be made a combined decision between the orthodontist and the layperson.

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Conflict Of Interest: Nil

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Influence of Commercially Available Herbal Mouthwash on the Surface Tomography of Two Different Types of Nickel Titanium Orthodontic Arch Wires – An in vitro Study

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ABSTRACT

Oral hygiene maintenance is one of the most important aspects to look after during orthodontic treatment. This can be achieved by the use of mechanical plaque controlling agents as well as chemical plaque controlling agents. However, chemical plaque controlling agents such as chemical mouthwashes cause damage to the surface tomography of the orthodontic arch wires and its surface coating. This reduces the working efficiency of the wire and also deteriorates the aesthetic component of these tooth colour coated orthodontic arch wires. Hence, the purpose of this study was to evaluate the effect of herbal mouthwash (Befresh) on the surface tomography of two different types of Nickel Titanium (NiTi) orthodontic arch wires. The study comprised of two groups: Group A (Test Group) and Group B (Control group). Each group consisted of two different wires: (i) a single 0.016 Copper (Cu) NiTi arch wire of 25mm length and (ii) a single 0.016 Teflon coated NiTi arch wire of 25mm length. Group A was immersed in Befresh mouthwash for 90 minutes while Group B was kept unaltered in room temperature without any manipulations. Both the groups were later viewed under Scanning electron microscope (SEM) and were qualitatively analysed. The results showed no significant changes in the surface tomography of the two arch wires, pre and post immersion into the herbal mouthwash (Befresh). Therefore, Befresh mouthwash can be prescribed by orthodontists as an adjuvant to chemical plaque controlling agent as it does not exhibit any detrimental effects to the surface tomography of the orthodontic arch wires.

KEY WORDS: BEFRESH HERBAL MOUTHWASH, CINNAMONUM ZEYLANICUM, SURFACE TOMOGRAPHY, SCANNING ELECTRON MICROSCOPE, TEFLON COATED NITI ARCHWIRE.

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INTRODUCTION

The human oral cavity houses millions of microorganisms, which are termed as commensals. Although harmless in general conditions, certain alterations in the conditions of the oral environment causes these microflorae to exert a deteriorating effect on the oral hard and soft tissue structures. These alterations may be in the form of dental caries, gingivitis, periodontitis, halitosis, mucosal lesions, etc (Zachrisson and Zachrisson, 1972). In spite of all the technological advances in today's world, there are studies that show inadequacy in the levels of mechanical oral hygiene practice (Sälzer et al., 2015). In fact, the World Health Organisation also says that these intraoral diseases are a health hazard globally, which may affect the systematic health of people in the long term (da Costa et al., 2017).

Orthodontic treatment is a form of dental mechanotherapy that utilizes wires, bands and brackets of different types of materials to bring about the movement of teeth from a malposed position to an ideal aesthetically acceptable position. The attachment of these components to the tooth surface leads to an increase in the accumulation of plaque (LUNDSTRöM and Hamp, 1980) . Increase in plaque accumulation leads to deterioration of the oral hygiene, increase in the microbial count and decrease in the pH of the oral cavity. These alterations in the oral environment is the primary reason for post orthodontic decalcification of teeth (CIANCIO, 1985; Tanner et al., 2012) .

Furthermore, the fixed orthodontic appliance act as a physical barrier that has to be overcome, in order to achieve ideal dental hygiene (Erbe et al., 2019). This facilitates the absolute need for the reinforcement of oral hygiene measures. Although mechanical methods of plaque control are of paramount importance, its efficacy also depends on the dexterity of the individual. Here, chemical plaque control methods serve as important adjuvants in orthodontic treatment phase (Erbe et al., 2019).

Chemical plaque controlling agents like chlorhexidine mouthwashes (CHX) reduce the microbial colony forming units (CFU) in the oral cavity, restore the balance in pH of the oral environment and also help in alleviating malodour. The property of substantivity exerted by the CHX mouthwash also has a prolonged antimicrobial effect in the oral cavity (Brightman et al., 1991). CHX mouthwash is also active against a variety aerobic, facultative anaerobic, yeasts, gram positive and gram negative organisms (Van der Weijden et al., 2015). However, these chemical mouthwashes also have some deteriorating effects in the oral cavity and are not recommended for long term use. Long term usage of CHX mouthwash is said to cause alteration of taste perception, cause staining of teeth and these preparations also have alcohol in it, that also alter the physical and chemical properties of the orthodontic components that are being used (Eliades and Athanasiou, 2002; Serrano et al., 2015).

Herbal mouthwashes are slowly emerging as a viable alternative to these chemical mouthwashes. They do not possess alcohol and neither to they cause any sort of harm to the surrounding oral structures on prolonged exposure (Dilipkumar et al., 2017). In fact, some of the herbal mouthwashes have also been found to have a soothing and anti-inflammatory effect on the gingival tissues. One such mouthwash is the Befresh herbal mouthwash. It is made up of cinnamon oil, eucalyptus oil, clove oil and spearmint oil. Cinnamon (*Cinnamonum zeylanicum*) exerts an antifungal and antibacterial effect. Clove oil has an additional property of acting as an antiseptic and an anaesthetic, and also exhibiting an astringent effect. Eucalyptus oil and spearmint oil, apart from exerting an antimicrobial effect, also help neutralising halitosis (Kripal, 2017). Also, this mouthwash is devoid of alcohol, which is not in case of the gold standard chlorhexidine (Kripal, 2017).

Many studies have been done on the effects of chemical mouthwash on several properties of orthodontic arch wires. 0.5% sodium fluoride containing mouthwash has been shown to increase the frictional resistance of almost all orthodontic brackets and arch wires (Geramy et al., 2017). Chemical mouthwashes have also been shown to be responsible for the ionic leaching of nickel and chromium ions (Mirhashemi et al., 2018). Even topical application of fluorides and fluoridated mouthwashes when treated with orthodontic arch wires show surface morphological changes (Gupta et al., 2018). At present, there is a need for an alternative to such deleterious chemical oral hygiene adjuvants, that deteriorate the properties of orthodontic appliances intraorally. Hence, the purpose of this study was to evaluate the effect of Befresh mouthwash on the surface tomography of orthodontic arch wires (Gupta et al., 2018).

MATERIAL AND METHODS

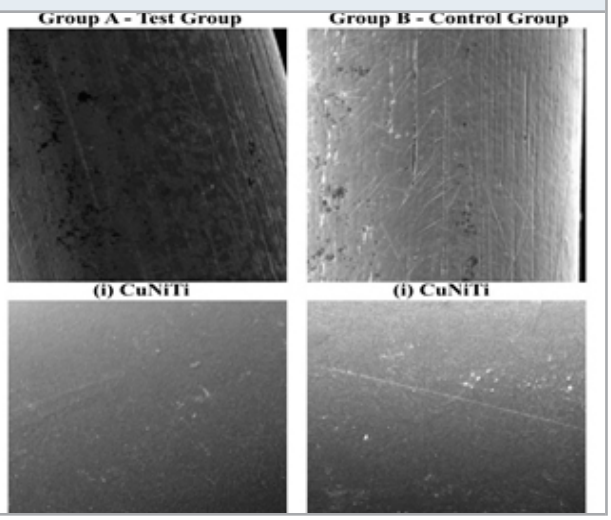
The study comprised of 2 groups, with each group comprising of two different types of 0.014 orthodontic archwires of 25mm length each. Group A was the Test Group, which consisted of (i) a 0.016 Copper (Cu) NiTi arch wire and (ii) a 0.016 Teflon coated NiTi arch wire, which was immersed in the Befresh herbal mouthwash. Group B was the Control Group, which consisted of (i) a 0.016 Copper (Cu) NiTi arch wire and (ii) a 0.016 Teflon coated NiTi arch wire, which was kept unaltered and stored at room temperature without any sort of manipulation.

Wires in Group A were immersed in the Befresh herbal mouthwash for a period of 90 minutes at room temperature. This exposure time is said to be equal to 3 months of exposure to routine 1-minute mouthwash usage (Walker et al., 2005). Wires in Group B were kept at room temperature without any manipulation. At the end of 90 minutes, the arch wires were removed from the mouthwash and rinsed thoroughly using distilled water. The archwires were kept to dry and were then subjected to qualitative analysis of the surface characteristics using a scanning electron microscope.

RESULTS AND DISCUSSION

The scanning electron microscopes were set to a magnification of 800x and the readings were evaluated. The CuNiTi and the Teflon coated NiTi arch wires showed certain linear areas, which can be attributed to the manufacturing process of that wire (Fig.1). There were no marked changes like pitting, mottling, globular patterns, smudges in the surface areas of both arch wires, indicating no obvious deformities in the tomography of both arch wires, pre and post immersion. Also, there was no loss of Teflon coating of the arch wire between Group A and B (Figure 1). This showed that the herbal mouthwash had no deteriorating effect on the surface characteristics as well as the surface coating of the orthodontic arch wires.

Figure 1: SEM image under 800x of Group A (Test Group) showing wire (i) CuNiTi and wire (ii) Teflon coated NiTi after immersion in Befresh mouthwash and Group B (Control Group) showing wire (i) CuNiTi and wire (ii) Teflon coated NiTi kept without any manipulations.



Oral hygiene maintenance is an important aspect of orthodontics as poor oral hygiene leads to poor gingival and periodontal health, which further affects tooth movement by altering the status of the bone (Schei et al., 1959). The components of fixed orthodontic appliances act as a scaffold onto which plaque gets accumulated, leading to the increase in the microflora. Bacteria found in the dental plaque have also been found to cause corrosion on metal surfaces (Kameda et al., 2014). Friction is a key determinant that influences the treatment duration and its outcome. Any sort of damage to the orthodontic components in the form of surface defects such as corrosion can hinder orthodontic tooth movement. This brings in the necessity for orthodontists to prescribe mouthwash, in order to maintain good oral hygiene as well as to preserve the integrity of the metallic components being used (Kameda et al., 2014).

Nickel titanium wires are the more commonly used wires for initial levelling and aligning procedures in orthodontic treatment. Since they make up for majority of treatment time, they were included as the wires of choice in this study. White spot lesions are one of the commonly occurring side effects of orthodontic therapy, if oral hygiene is not well maintained. This makes it necessary to prescribe a fluoride containing mouthwash for remineralization of the tooth (Khoroushi and Kachuie, 2017). Fluoride containing mouthwashes have been seen to cause surface changes in orthodontic archwires and brackets. Furthermore, fluoride containing mouthwashes cause leaching of metallic ions from the metallic components of the fixed orthodontic appliances, that alter the taste perception and also discolour the teeth (Aghili et al., 2017). Corrosion of orthodontic archwire is another drawback seen in the usage of alcohol containing fluoride mouthwashes. This is mainly because of its interference with the passivating layer of the orthodontic archwires (Huang, 2002). Studies show that fluoride containing mouthwashes cause reduction in the corrosion resistance of nickel titanium and stainless-steel wires.

This is directly proportional to the fluoride concentration in the mouthwash (Heravi et al., 2015). Study conducted by Geramy et al., (2017) shows that friction rate is increased in wires treated with fluoride mouthwashes. Although chlorhexidine is considered as the gold standard of mouthwashes, it cannot be prescribed for longer duration as it causes certain side effects. It causes staining and alteration of taste perception, imparting a metallic taste on prolonged use (Dolles et al., 1979). Study conducted by Danaei et al., (2011) shows that chlorhexidine mouthwash causes ion release from stainless steel orthodontic brackets. Alcohol containing chemical mouthwashes like Listerine are also not recommended in case of aesthetic coated archwires, as they have a detrimental effect on the surface coating (Hussein and Ghaib, 2017).

Herbal mouthwashes on the other hand are alcohol free and have been used for many years. Herbal derivates have no side effects on prolonged usage, are cost effective and easier to manufacture. Kripal et al., in his study shows Befresh mouthwash to be equivalent in efficiency to that of chlorhexidine mouthwash, in terms of antimicrobial activity (Kripal, 2017). Study conducted by Brar et al., has shown that Listerine and Chlorhexidine mouthwashes have more corrosive effect on the wire as compared to an organic herbal (neem) mouthwash (Brar et al., 2015). Furthermore, another study showed ion release in metal brackets and wires to be higher in the chlorhexidine group (Danaei et al., 2011). The reason for this is believed to be the higher acidic pH of chlorhexidine which is believed to be 6.5 as compared to the 7.75 pH of the organic herbal mouthwashes (Brar et al., 2015). Even though herbal mouthwash is not as effective as chlorhexidine mouthwash in similar concentration, their efficacy can be well augmented with the increase in its dosage and exposure time (Parwani et al., 2013). This is not possible in case of chemical mouthwashes, owing to their added side effects which do not permit long term exposure (Kripal, 2017).

Chemical mouthwashes are also contraindicated in cases which involve aesthetic arch wires. The epoxy coating of the arch wire gets disintegrated in the presence of alcohol (Mohsin and Al-Sheakli, 2016). However, this is not the case for organic mouthwashes, which are devoid of alcohol. This gives rise to fewer complications and side effects (Jongsma et al., 2015). Usage of herbal mouthwash can prove to be a valuable adjuvant to mechanical plaque controlling methods, as its reduced cytotoxicity, fewer to none detrimental effect on the orthodontic components and its antimicrobial activity puts it above the chemical mouthwashes. Although chlorhexidine is still considered to be the gold standard of mouthwash, herbal mouthwash can be an effective replacement. This is especially true in orthodontic cases, which warrant the use of mouthwashes for the longer run. This not only solves the purpose that the chemical mouthwashes were made for, it is also compatible with the treatment needs from an orthodontist's perspective. The physical properties as well as the aesthetic colour stability of the orthodontic components are well preserved. Since only one type of wire has been used in this study, there is a need to further evaluate the effect of herbal mouthwash on other types of orthodontic arch wires, which would be the scope for future studies.

CONCLUSION

The organic herbal mouthwashes like Befresh mouthwash, are a valuable and effective alternative to chemical mouthwash. They do not cause any changes to the surface tomography of orthodontic arch wires and orthodontic brackets. They are economic, environment friendly and are more accepted by patients owing to their natural taste and fragrance. They also have fever to no side effects as compared to chemical mouthwashes, which are deleterious on long term usage. Although herbal mouthwash is not as effective as chemical mouthwash in its antimicrobial efficacy, it could serve as an ideal adjuvant to the chemical plaque controlling agent as it is less deteriorating even in higher dosage and prolonged use, which would also increase its antimicrobial activity at higher dosage and exposure.

This is not the case with chemical mouthwash, without risking their deteriorating side effects. The results of this study have shown the influence of herbal mouthwash on the surface tomography of the CuNiTi orthodontic arch wires and also the surface coating of aesthetic NiTi arch wires. Further studies are required to look into the influence of herbal mouthwash on the physical characteristics of orthodontic arch wires and other components, which would be the scope for future studies.

Authors Contribution: All authors have equal contribution in bringing out this research work.

Conflict Of Interest: None.

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Analysis of Hypoglycemic and Anti-Oxidative Potential of *Gymnema sylvestre* Ethanolic Extract in Alloxan Induced Diabetic Rats

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ABSTRACT

Diabetes is a chronic metabolic disorder characterized by prolonged hyperglycemia (Fasting plasma glucose >126mg/dl) which is affecting 425 million people worldwide. Prolonged hyperglycemia leads to formation of AGEs by glycosylating macromolecules leads to oxidative stress in tissues. Presents study investigates the effect of ethanolic extract of *Gymnema sylvestre* (500 mg/kg b.wt) on Alloxan (100 mg/kg b.wt) induced diabetic rats. Rats were dived into five groups namely, normal control, diabetic control, diabetic rats administered with the extract for 10, 20, and 30 days respectively. Standard protocol was followed to measure the concentration of the parameters, and statistical analysis was done by Tukey multiple range test compared with the entire column after ANOVA using Prism Graph Pad. The level of significance is represented for diabetic VS treated group having p value *** $p<0.05$, **$p<0.01$, *$p<0.001$, Ns-Non significant, >0.05. Plasma glucose, Glutathione (GSH), Glutathione s transferase (GST), Glutathione peroxidase (GPx), Total thiol (T-SH), Catalase, Superoxide dismutase (SOD), and Ascorbate (Vitamin C) in the tissue sample of Liver, Kidney, Heart of the rats were probed. Result showed significant restoration of plasma glucose and tissue anti- oxidative enzymes activities after extract administration to diabetic rats for 30 days. The research strongly supports exogenous intake of *Gymnema sylvestre* leaf extract in diabetes would ameliorate the damaged caused by hyperglycemia and oxidative stress. However extensive research is required for the further validation of the efficacy of the plant.

KEY WORDS: DIABETES, ANTI-OXIDANT, ROS, AGES, HYPERGLYCEMIA.

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INTRODUCTION

Type 2 Diabetes is a chronic metabolic disorder with marked hyperglycemia, reduced insulin sensitivity, increased oxidative stress and altered carbohydrate, fat, and protein metabolism. There are currently 425 million people with diabetes worldwide, and this number is expected to reach 629 million by 2045, with Type 2 Diabetes Mellitus (T2DM) being the most expressive form of the disease Current evidence has suggested that oxidative stress due to reactive oxygen species (ROS) and nitrogen species plays an important role in the pathogenesis of chronic diseases such as type 2 Diabetes mellitus (Ahmad et al., 2017). Prolonged hyperglycemia accompanied with higher reactive oxygen species (ROS) may develop into macro and microvascular diseases which are the main causes of morbidity and mortality worldwide associated with T2DM (Goycheva et al., 2017). ROS in association with hyperglycemia enhances four major molecular pathways pertaining to tissue damage. The four major pathways are increased hexosamine flux, activation of Protein Kinase C pathways (PKC), enhanced polyol pathways and increased advanced glycation end product (AGEs) (Ahmad et al., 2017, IDF, 2017 and ADA, 2018).

Monosaccharide such as fructose, glucose, glyceraldehyde etc, reacts non-enzymatically with an amino group of protein, lipid, and nucleic acid to form nascent macromolecule which is termed as advanced glycation end product (AGEs), which is hardly metabolized or expelled from the body. Formation of AGE results in interaction with receptor of AGEs namely Receptor of advanced glycation end product (RAGE) which mediates activation of cytosolic intermediates for the formation of ROS (Ahmad et al., 2017). To neutralize the reactive oxygen species animal body is equipped with various types of Enzymatic (Catalase, Superoxide dismutase, GPX, GST, etc) and non-enzymatic (Ascorbate, Tocopherol, lycopene, beta carotene, glutathione, etc) antioxidant molecules.

The antioxidant molecule scavenges the various types of radicals into molecular oxygen and other essential components which don't harm to tissues (York-Duran et al., 2019). Oxidative stress has a significant role in further complications of type 2 Diabetes Mellitus. An increase in ROS level leads to elevated or dysregulated production of antioxidants like catalase, superoxide dismutase, glutathione peroxidase, etc. The variation in the availability of the mentioned parameters pronounces the tissue susceptibility to the oxidative stress pertaining to the appearance of diabetes and its associated complications (He et al., 2017). This enhanced oxidative stress causes severe tissue damage in the liver, kidney, brain, heart, Pancreas etc, because of a lack of counterbalance between increased ROS and antioxidant. This imbalance could be nullified by an exogenous supply of anti-oxidant and hypoglycemic botanicals (Jha et al., 2016; Wilson et al., 2018 Seo et al., 2019).

The present research is aims to reveal the potential of the *Gymnema sylvestre* herbal extract to nullify the complication of the Diabetes. The belief for the use of plant-based product is still a primary option in Indian subcontinent. In such case phytotherapy becomes the relevant topic to probe a natural diabetic treatment naturally because it contains both active ingredient as well as anti-oxidant to cater the need to reduce glucose use to combat AGEs and its related complications (Kajal and Singh, 2018).

The desired plant, *Gymnema sylvestre* is a large woody climber running over the tops of high trees and belongs to the family Asclepiadaceae. The vernacular names of G. sylvestre are in English-Periploca of the woods, Hindi-Gurmar, Telugu-Podapatri, and Sanskrit- AjaboUi. The plant is an herb native to the tropical forests of southern and central India and Sri Lanka (Pham et al., 2018). *Wistar norvegicus* male rats were included in the experiment and diabetogenic material was Alloxan because it is widely used to create an experimental diabetic model in rats due to its selective destruction of pancreatic β cell because of its free radical production upon decomposition. It exhibits multiphasic changes in blood glucose, insulin concentration accompanied by histo-architectural changes pertaining to necrotic cell death (Radenkovic et al., 2016). There is a lack of literature availability in the organ tissue oxidative stress caused due to diabetes and its amelioration by *Gymnema sylvestre*. The present study examines the efficacy and potential of the desired plant in reduction of oxidative stress in different organs and its hypoglycemic properties in diabetic subjects (Pham et al., 2018).

MATERIAL AND METHODS

For the present research work healthy male wistar rats (*Rattus norvegicus*) of weight ranging 180-200 gram were selected and provided ambient physical and physiological condition as per the standard protocol and all the experimental protocol was carried based on the guideline adopted by Mahavir Cancer Sansthan ethical comittee, Phulwari sariff Patna.

Induction of diabetes: Diabetes was induced by repeated dose of Alloxan monohydrate 100 mg/kg b.wt in cold citrate buffer bearing pH 4.5.

Plant materials Leaves of *Gymnema sylvestre*

Preparation of herbal extract: Freshly harvested plant samples were washed under running tap water blotted with filter paper and was dried in the shade at room temperature. The dried plant materials i.e., leaf of *Gymnema sylvestre* was subjected to converted into fine powder which was then soaked with absolute ethanol and kept in dark for 48 hours. After that the entire extract of plant was filtered using Whatman filter paper till the clear material appeared. The solvent containing secondary metabolite of the plant was mounted on the vacuum rotary evaporator at 40 ^{0}C. The extract was kept on the vacuum rotary till the thick paste appeared devoid of any solvent material. The thick paste colloidal

was lyophillysed in lyophyliser (Labconco, USA). The lyophilized plant extract was stored in deep freezer at _80 ^{0}C until further test.

Sample collection: After the treatment of the extract for 10, 20, and 30 days respectively the tissues (Liver, Kidney, and heart) collected were for anti-oxidant quantification. For anti-oxidant analysis the tissue sample were subjected to preparation of post mitochondrial supernatant (PMS).

Chemicals and reagents: All the reagents were prepared in the laboratory using high grade chemical. Glucose estimation was done by GOD POD method and the estimation of the antioxidant parameters were carried out by the published standard literature like Estimation Of Catalase was done by (Sinha AK, 1972), estimation of total reduced Glutathione (GSH) (Boyne and Ellman, 1972), estimation of Ascorbic acid (Newman et al., 2000; Omaye et al., 1979), estimation of Glutathione Peroxidase (Rostruck et al., 1979), estimation of Glutathione-s-transferase by (Habig et al., 1974), and quantification of Superoxide dismutase (SOD) was done by (Marklund S and Marklund G, 1974).

RESULTS AND DISCUSSION

Recent reports of chemotherapeutics resistant in treatment of diabetes mellitus have forced the research community to look towards nature for the better remedy and hence the herbal drugs are emerging as a future hope in control of diabetes mellitus.

Effect of *Gymnema sylvestre* ethanolic extract on fasting plasma in the groups: Plasma blood glucose level plays a vital role in complications associated with diabetes. In the present study Diabetic rats registered nearly four times elevated plasma glucose level. Decrease in blood glucose level was associated with alleviation of oxidative stress by herbal extract. *Gymnema sylvestre* treated rats recovered glucose concentration to 73.40% (Table1) which is in parallel with previous study (Khan et al., 2019).

Effect of *Gymnema sylvestre* ethanolic extract on Glutathione (GSH) in the Liver, Kidney and Heart in the groups: The present study is aimed to probe the oxidative stress in different tissues like liver, kidney, brain and heart after Alloxan induced diabetes and its treatment through herbal extract. Alloxan induced diabetic rats was reported to increase oxidative stress which is reflected by dysregulated peroxide, superoxide, hydroxyl radical etc (Aluwong et al., 2016). In the present investigation, Gymnema sylvestre showed significant recovery in reduced glutathione level in organs investigated (liver 77%, kidney 85.71%, and heart 88.45%) (Table 2-4) (Khan et al., 2019).

Effect of *Gymnema sylvestre* ethanolic extract on Glutathione peroxidase (GPx), and catalase (CAT) in the Liver, Kidney and Heart in the groups: For the assessment of peroxide radical level, Glutathione peroxidase and catalase quantification were performed. Lowered Glutathione peroxidase and catalase level signifies high peroxide stress in Diabetic subjects due to less availability of the related enzymes (Kaskoos et al., 2015). *Gymnema sylvestre* significantly restored the activity of glutathione peroxidase towards normal range (liver 90%, kidney 94%, and heart 86 %.) (Table 2-4) which was accompanied by significant recovery of catalase in the tissues after treatment of the animal with the extract of *Gymnema sylvestre* (liver 67.89%, kidney 85.18%, and heart 88.02%) (Table II-IV). The result obtained was as par with the findings (Porkodi et al., 2020).

Table 1. Estimation of fasting plasma glucose in *Gymnema sylvestre* treated Diabetic rats

	Fasting Plasma Glucose (mg/dl)			
Control	Alloxan treated	Diabetic 10 Days GSE Treated	Diabetic 20 Days GSE Treated	Diabetic 30 Days GSE Treated
90.00±7.90*	368.0±14.40*	264.0±20.74*	196.0±11.40*	122.0±7.82*

Table represents fluctuation in the glucose concentration in the classified groups. The diabetic rats showed significant recovery after treatment with TCE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range tests compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *p<0.001.

Effect of *Gymnema sylvestre* ethanolic extract on Glutathione s transferase (GST), Superoxide dismutase (SOD) in the Liver, Kidney and Heart in the groups: Glutathione s transferase catalyses the conjugation of glutathione (GSH) to different varieties of endogenous and exogenous electrophilic components (Ghosh et al., 2018). The investigation showed, there was more than 50% reduction in the concentration of Glutathione S

transferase in diabetes as compared to normal control. Rats treated with *Gymnema sylvestre* ethanolic extract showed significant recovery in (liver 74.17%, kidney 81.10%, and heart 76.31%) (Table 2-4). The results obtained were in parallel with the findings (Khan et al., 2019).

Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion (O2-) into H_2O_2 and molecular oxygen (Wang et al., 2018). Diabetes rats treated with *Gymnema sylvestre* extract showed significant recovery (liver 65.53%, kidney 78.20%, and heart 83.45%) (Table 5-7) and this study was in accordance with the findings of (Priya et al., 2017).

Table 2. Estimation of Glutathione Content and Enzyme Activity of Glutathione Peroxidase **(GSH-Px), Glutathione-S-transferase (GST), a Catalase in Liver PMS of Gymnema sylvestre treated diabetic wistar rat**

	Enzyme activity			
	Glutathione content (µg/mL)	GSH-Px (nmol/NADPH oxidized/min)	GST (units/mg protein/min)	Catalase (mU/mg protein)
Control	218.8±12.09*	2.33±0.17*	182.0±10.37*	325.5±7.19*
Alloxan treated	77.95±5.10*	0.11±0.00*	74.10±10.39*	102.2±8.65*
Diabetic 10 Days GSE Treated	0.32±0.00 ns	87.60±7.09***	140.0±11.18 ns	170.2±14.59 ns
Diabetic 20 Days GSE Treated	86.79±9.60*	1.2±0.12*	113.6±7.40*	188.2±7.49*
Diabetic 30 Days GSE Treated	136.4±6.54*	2.11±0.17*	135.2±7.59*	221.0±49.15*

Table represents fluctuation in the mentioned parameters in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *** p<0.05, **p<0.01, *p<0.0001, Ns Non significant, >0.05.

Table 3. Estimation of Glutathione Content and Enzyme Activity of Glutathione Peroxidase **(GSH-Px), Glutathione-S-transferase (GST), a Catalase in Kidney PMS of Gymnema sylvestre treated diabetic wistar rat**

	Enzyme activity			
	Glutathione content (µg/mL)	GSH-Px (nmol/NADPH oxidized/min)	GST (units/mg protein/min)	Catalase (mU/mg protein)
Control	140.4±7.62*	3.05±0.27*	85.88±6.42*	243.4±5.76*
Alloxan treated	72.68±10.32*	0.61±0.11*	39.84±3.70*	116.5±11.73*
Diabetic 10 Days GSE Treated	83.56±4.358***	1.30±0.21*	52.46±5.30**	141.0±8.21**
Diabetic 20 Days GSE Treated	98.30±5.03*	2.30±0.4*	61.60±6.42*	180.0±7.90*
Diabetic 30 Days GSE Treated	120.3±7.91*	2.89±0.10*	70.40±3.57*	207.0±10.37*

Table represents fluctuation in the mentioned parameters in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *** p<0.05, **p<0.01, *p<0.0001, Ns Non significant, >0.05.

Table 4. Estimation of Glutathione Content and Enzyme Activity of Glutathione Peroxidase (GSH-Px), Glutathione-S-transferase (GST), a Catalase in Heart PMS of *Gymnema sylvestre* treated diabetic wistar rat

	Enzyme activity			
	Glutathione content (µg/mL)	GSH-Px (nmol/NADPH oxidized/min)	GST (units/mg protein/min)	Catalase (mU/mg protein)
Control	125.9±7.590*	3.05±0.27*	85.88±6.42*	243.4±5.76*
Alloxan treated	67.45±6.028*	0.61±0.11*	39.84±3.70*	116.5±11.73*
Diabetic 10 Days GSE Treated	77.73±5.760***	1.30±0.21**	52.46±5.30***	141.0±8.21 ns
Diabetic 20 Days GSE Treated	87.21±7.900*	2.30±0.4*	61.60±6.42*	180.0±7.90***
Diabetic 30 Days GSE Treated	100.9±7.872*	2.89±0.10*	70.40±3.57*	207.0±10.37*

Table represents fluctuation in the mentioned parameters in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *** p<0.05, **p<0.01, *p<0.0001, Ns Non significant, >0.05

Table 5. Estimation of Glutathione Content and Enzyme Activity of Ascorbic Acid, Total Thiol (TSH), superoxide dismutase (SOD) in Liver PMS of *Gymnema sylvestre* treated diabetic wistar rat.

	Enzyme activity		
	Ascorbic Acid (mg/dl)	Total thiol (TSH) (nmol/NADPH oxidized/min)	SOD (units/mg protein/min)
Control	3.139±0.09*	11.19±0.82*	5.42±0.60*
Alloxan Treated	1.084±0.18*	2.136±0.25*	16.05±0.75*
Diabetes 10 days GSE Treated	1.646±0.16*	4.240±0.39**	13.36±0.40
Diabetes 20 days GSE Treated	2.420±0.30*	6.034±0.37*	10.84±0.59*
Diabetes 30 days GSE Treated	2.99±0.08*	8.482±1.19*	8.27±0.73*

significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *** p<0.05, **p<0.01, *p<0.0001, Ns Non significant, >0.05

Effect of *Gymnema sylvestre* ethanolic extract on Total Thiol (T-SH) and Ascorbate (Vitamin C) in the Liver, Kidney and Heart in the groups: Total thiol contents give an idea of oxidative stress and status of the other enzymatic parameters because most of the enzymes have sulphadryl group in their active site (Ates et al., 2015). Diabetic rats treated with *Gymnema sylvestre* extract regained total thiol concentration to nearly 75.78% in liver, 91% in kidney, 82%, and 60.58% in heart after 30 days of extract administration (Table 5-7) and is in accordance with the findings of (Requejo et al., 2010). Vitamin C plays a vital role against oxidative

stress and helps to overcome it (Spoelstra et al., 2018). Phyto-extract administration has led to increase in tissue Ascorbate. Treatment with ethanolic extract of *Gymnema sylvestre* showed significant recovery in organs under investigation (liver liver 95.5%, kidney 89.37%, and heart 88.32%) (Table 5-7). Increase in vitamin C after drug administration reduced prolonged hyperglycemic induced oxidative stress and the study found itself as par with the findings of (Madani et al., 2015).

Table 6. Estimation of Ascorbic Acid, Total Thiol (TSH), superoxide dismutase (SOD) in Kidney PMS of Gymnema sylvestre treated diabetic wistar rat.

	Enzyme activity		
	Ascorbic Acid (mg/dl)	Total thiol (TSH) (nmol/NADPH oxidized/min)	SOD (units/mg) protein/min
Control	3.202±0.21*	5.640±0.53*	3.66±0.52*
Alloxan Treated	0.7240±0.12*	2.438±0.22*	9.0±0.79*
Diabetes 10 days GSE Treated	1.818±0.26*	3.416±0.33*	7.23±0.49*
Diabetes 20 days GSE Treated	2.418±0.22*	3.976±0.31*	5.92±0.55*
Diabetes 30 days GSE Treated	2.864±0.13*	5.106±0.30*	4.68±0.42***

Table represents fluctuation in the mentioned parameters in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *** p<0.05, **p<0.01, *p<0.0001, Ns Non significant, >0.05

Table 7. Estimation of Ascorbic Acid, Total Thiol (TSH), superoxide dismutase (SOD) in Heart PMS of *Gymnema sylvestre* treated diabetic wistar rat.

	Enzyme activity		
	Ascorbic Acid (mg/dl)	Total thiol (TSH) (nmol/NADPH oxidized/min)	SOD (units/mg protein/min)
Control	2.742±0.14*	5.150±0.80*	3.48±0.39*
Alloxan Treated	1.192±0.06*	0.724±0.09*	6.22±0.53*
Diabetes 10 days GSE Treated	1.872±0.09*	1.842±0.20*	5.33±0.39*
Diabetes 20 days GSE Treated	2.192±0.07*	2.280±0.21*	4.80±0.26*
Diabetes 30 days GSE Treated	2.424±0.08*	3.090±0.31*	4.17±0.32***

Table represents fluctuation in the mentioned parameters in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value *** p<0.05, **p<0.01, *p<0.0001, Ns Non significant, >0.05

CONCLUSION

Diabetes is a multifactorial disorder which affects the individual in many ways. Nephropathy, cardiovascular complication, intestinal disorder, hepatic damage, retinopathy, and neuropathy are some of the glimpse of the Diabetes complications. And behind each disorder macromolecule glycosylation and free radical induced tissue damages are the key ingredients. The present study reveals that the *Gymnema sylvestre* ethanolic extract as an important herbal drug in alleviation of the fasting plasma glucose and the oxidative stress significantly. Reduction in the oxidative stress in the tissues of the liver, kidney, and heart and hypoglycemic activities pronounces the effectiveness of the herb. With the increase in diabetes population at an alarming rate demands an urgent need for more effective research and assessment to find out the active phytochemical ingredients with exact active anti-diabetic mechanism. In this regard, a long and continuous research accompanied with large sample size, and translational study is required.

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Characterization of Antimycobacterial Activity of Bacteriocins Isolated from Fish-Gut Associated Lactic Acid Bacteria

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ABSTRACT

Tuberculosis (TB) is a contagious airborne disease caused by the pathogen, *Mycobacterium tuberculosis* (MTB). The conventional anti-tubercular drugs such as isoniazid and rifampicin have maximum activity and lengthy duration of therapy. The risk of serious adverse events such as hepatotoxicity, discourage both patients and providers. The big challenge here has been to find a new drug effective against TB. Lactic acid bacteria (LAB) are widely distributed in dairy products and fermented foods and also from non-dairy sources. LAB would produce novel antimicrobial peptides (bacteriocins) with unique structural characteristics and applications. The antimycobacterial properties of bacteriocins isolated from lactic acid bacteria have been studied. In this study, we isolated lactic acid bacteria from fish gut and evaluated their bacteriocins for antimycobacterial activity. Different genus of LAB was isolated and characterized viz. *Pediococcus sp., Aerococcus sp, Lactobacillus sp*. from fish gut. All the isolates were screened for their antibacterial and antimycobacterial activity. Based on the activity against *M. smegmatis* MC^2155, *Lactobacillus spp*. (BFO21) was selected and characterized by 16S rRNA gene sequence analysis. Bacteriocin were isolated from *Lactobacillus plantarum* BFO21 and partially purified using chloroform solvent extraction method. About 98% of RLU reduction in terms of inhibition was found with bacteriocins of *L. plantarum* BFO21 against *M. tuberculosis* H37Rv through LRP assay. According to our results, the isolate *Lactobacillus plantarum* BFO21 should be evaluated for further characterization of its bacteriocins to explore their anti-tubercular activity against both replicating drug resistant *M. tuberculosis* and non-replicating *M. tuberculosis* (Latent TB)..

KEY WORDS: TUBERCULOSIS; BACTERIOCINS; FISH GUT; LACTOBACILLUS; ANTI-TUBERCULAR ACTIVITY; LRP ASSAY.

ARTICLE INFORMATION

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INTRODUCTION

Tuberculosis (TB) poses serious epidemics around the world caused by the pathogen *Mycobacterium tuberculosis* (Mtb). The increasing rate of HIV-related TB, multi-drug resistant TB (MDR- TB) and Extensively Drug Resistant TB (XDR-TB) also concerned globally. Treatment for tuberculosis requires 6-8 months for new cases and 18-24 months for MDR TB whereas the treatment for XDR-TB is ineffective making the treatment options seriously limited. The big challenge here has been to find new drugs effective against tuberculosis. In this context, antimicrobial peptides such as bacteriocins produced by lactic acid bacteria has been emphasized for their prominent antimycobacterial properties due to cationic characteristics which likely binds to the anionic lipids in membrane (Sivaraj et al., 2018). There are only few studies carried out on bacteriocins focused on their antimicrobial or antitubercular activity but many studies focused on their potential applications as food preservatives (Perez et al., 2018). Bacteriocins exhibit significant potency against multidrug-resistant bacteria and also offer promising lead compound as substitutes or conjugates to current therapeutic compounds (Meade et al., 2020).

The concept of research on bacteriocins applications are expanding from food preservative to human health including novel drug delivery systems, anti-tubercular treatment and anticancer treatment applications (Meade et al., 2020). Lactic Acid Bacteria (LAB) is a group of Gram positive, catalase negative, often non-motile organisms that are grouped in to two distinct phyla, such as Firmicutes and Actinobacteria. Around 15 different genera of LAB have been reported and some of them are dominant genera viz. *Lactobacillus, Streptococcus, Pediococcus, Enterococcus, Aerococcus*, etc. LAB have been isolated from various sources like raw milk, cultured milk products, meat products, fish, grains, green plants, fermenting vegetables, mucosal surface of animals (Lindgren and Dobogosz, 1990). Fish gut is considered as complex ecosystem that contains numerous microorganisms (Wong et al., 2013). The microbiota of fishes helps in antagonistic activity against pathogens and is in various immune responses (Huber et al. 2004). LAB isolated from the gastrointestinal tract (GIT) of fishes had been reported to have the potential as probiotic agents by protecting the aquatic species from various aquatic infections and also by killing the pathogens involved in the spoilage of fish products (Meade et al., 2020).

Broad range of various lactic acid bacteria species found in the GIT of various fish species which include both fresh water and marine water species (Merrifield et al., 2014). It was believed that LAB obtained from the GIT of fishes has the potential to develop as ideal probiotic agents (Gomez-Sala et al., 2015). LAB is considered as "Generally Recognized as Safe (GRAS)" microorganisms, which produce various compounds during lactic acid fermentation including organic acids, diacetyl hydrogen peroxide, bacteriocins, etc. Bacteriocins produced by LAB are considered as small peptides (<10 kDa) that have greater antibacterial activity by means of their unique characteristics such as cationic in nature, heat-stable, amphiphilic and adsorption to the gram-positive cell surfaces. LAB and their metabolic products usages are generally considered as safe (Zacharof and Lovitt, 2012). LAB-bacteriocins are emerging as a novel alternative to antibiotics and known to exert either bacteriostatic or bactericidal activity toward sensitive organisms. Bacteriocins of LAB target specific species and do not affect other population within the same ecosystem (Vieco-Saiz et al., 2019).

Bacteriocins are secreted in the logarithmic growth phase of bacteria with increasing bacterial numbers and optimal culture conditions promoting increasing peptide secretion (Ge et al., 2019; Anbarasu et al., 2020). Bacteriocins are extracellularly released peptides and they are mainly categorized into class I, class II, class III and class IV based on the host producer, molecular weight and amino acid sequence (Meade et al., 2020). The electrostatic interaction between positive charge of bacteriocins and the negative charge of bacterial cell membranes plays a significant role in the initial interaction thereby facilitating the binding of the molecules to the membranes of target cells (Perez et al., 2015). LAB isolated from GI of estuarine fish and freshwater fishes have showed significant antimicrobial activity against various aquaculture pathogens (Sahoo et al., 2015; Hagi et al.,2004; Ghosh et al., 2014).

The antimycobacterial properties of bacteriocins isolated from lactic acid bacteria have been studied by few researchers globally. Lantibiotics are class I bacteriocins that certainly possess sufficient potential for treating tuberculosis in future. Bacteriocins from lactic acid bacteria have showed greater antimycobacterial activity than equal concentrations of rifampicin in vitro model (Carroll and Jim O'Mahony, 2011; Sosunov et al., 2007). Pérez et al., (2018) demonstrated synergistic actions of enterocin AS-48 (bacteriocins produced by LAB) with ethambutol against *Mycobacterium tuberculosis* and revealed its potential role in tuberculosis treatment. In this context, the present study was aimed to isolate lactic acid bacteria from fish gut and to evaluate their bacteriocins for anti-tubercular activity against *M. tuberculosis* using luciferase reporter phage (LRP) assay.

MATERIAL AND METHODS

The fishes such as *Rastrelliger kanagurta*, *Tilapia, Centropristis striata* and *Teuthida* were aseptically dissected to collect intestinal contents, weighed and homogenized. They were each transferred aseptically to 10 ml of saline, shaken well and about 1 ml aliquots were transferred to 10ml of MRS broth each and incubated at 30˚C for 18 hours. After incubation, ten-fold serial dilution of samples was carried out and plated onto MRS agar containing bromocresol (0.04mg/ml) and incubated at 30˚C for 24 hours. Colonies showing yellow zones

were selected and sub-cultured on to MRS agar plate. Isolates that were catalase negative and gram positive further got identified at genus level according to method described by Nikita and Hemangi (2012) and Kalschne et al., (2015).

To test the antimicrobial and antimycobacterial activities, one ml of overnight grown LAB culture was transferred to 100 ml of MRS broth (Himedia, India) and incubated at 30˚C in shaker (100rpm). After 18 hours of incubation, cell free supernatant (CFS) was collected by centrifugation at 5000rpm for 10mins (4˚C). Then pH of CFS was adjusted to 6.5 and treated with catalase (1mg/ml) for testing their inhibitory activity against non-mycobacterial strains such as *S. aureus, B. cereus, E. coli* and *K. pnemuoniae* and mycobacterial strains, *M. smegamtis* mc2155 by agar well diffusion assay (Anbarasu et al., 2019). Briefly, about 5 mm diameter wells were cut on nutrient agar medium containing non-mycobacterial cultures and middlebrook 7H9 agar plate containing *M. smegamtis* mc2155. About 100µl of treated CFS from all isolates were added into each well. Nutrient agar plates were incubated at 30˚C for 18 hours and middlebrook 7H9 agar plates were incubated for 48 hours at 37˚C. Zones of inhibition were measured in mm.

LAB isolate (BFO21) showing activity against *M. smegmatis* mc2155 was identified by 16S rRNA gene sequencing. Briefly, genomic DNA was isolated using a modified phenol-chloroform protocol. PCR was performed using the following primer pair-forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1492R (5'-TAC GGT TAC CTT GTT ACG ACT T-3') at the following conditions: initial denaturation (97°C for 5 min followed by 35 cycles at 94°C for 2 min), annealing (51°C for 1 min) extension (72°C for 2 min) and final extension (72 °C for 5 min). PCR products were purified and subjected to sanger sequencing for 16s rRNA gene sequence analysis. The homology of the obtained sequence was analyzed using NCBI BLAST.

Table 1a. Isolation and characterization of lactic acid bacteria from fish gut

S. No	Reference ID	Source (Fish)	Morphology	Gas Production	Growth @10˚C	Growth @45˚C	Growth @pH 4.4	Growth @pH 9.6	Growth @ 6.5% NaCl	Growth @ 18% NaCl
1	BFO21	*Tilapia*	Gram positive bacilli	–	–	+	+	+	+	+
2	BFO22	*Tilapia*	Gram positive bacilli	–	+	+	+	+	+	+
3	BFO27	*Rastrelliger kanagurta*	Gram positive cocci (tetrads)	–	–	–	–	+	+	–
4	BFO28	*Rastrelliger kanagurta*	Gram positive cocci (tetrads)	–	+	+	–	+	–	–
5	BFO29	*Centropristis striata*	Gram positive cocci (tetrads)	–	+	+	+	+	+	–
6	BFO30	*Teuthida*	Gram positive cocci (tetrads)	–	+	+	+	+	–	–

Table 1b. LAB identified at genus level by biochemical methods

S. No	Reference ID	Genus Identified
1	BFO21	*Lactobacillus sp.*
2	BFO22	*Lactobacillus sp.*
3	BFO27	*Streptococcus sp.*
4	BFO28	*Aerococcus sp.*
5	BFO29	*Pediococcus sp.*
6	BFO30	*Pediococcus sp.*

Table 2. Inhibitory activity of cell free supernatant by agar well diffusion assay

S. No	Reference ID	Zone of Inhibition (mm)				
		S. aureus	*B. cereus*	*E. coli*	*K. pneumoniae*	*M. smegmatis*
1	BFO21	-	11	-	-	12
2	BFO22	-	10	-	-	-
3	BFO27	-	11	-	-	-
4	BFO28	-	-	-	-	-
5	BFO29	-	8	-	-	-
6	BFO30	-	10	-	-	-

Lactobacillus spp. (BFO21) was subjected to partial purification of bacteriocin using solvent extraction method. Briefly, overnight grown LAB isolate was inoculated to 500ml of MRS broth and incubated at 30°C for 18 hours in shaker, 100 rpm. CFS were collected by centrifugation at 5000 rpm for 15minutes at 4°C, added with equal amount of chloroform and kept in magnetic stirrer for 20minutes at 4°C. Resulting white precipitate was collected by centrifugation at 5000rpm for 30 minutes, lyophilized and stored in PBS buffer

(pH 6.0) at -20°C. The precipitated bacteriocin was filtered through 0.45micron syringe driven filter and subjected to antimycobacterial screening.

Table 3. Anti-tubercular activity of partially purified bacteriocin by LRP assay

S. No	Reference ID	Percentage of Reduction in RLU	Result
1	Rifampicin	91.93	Inhibition
2	BFO21*	98.00	Inhibition

*Bacteriocins produced by *L. plantarum*.

To screen for anti-tubercular activity of partially purified bacteriocin against *M. tuberculosis* H37Rv, LRP assay was used as mentioned (Anbarasu et al., 2019). Briefly, 400µl of middelbrook 7H9 broth was added to two cryo vials (Control), 400µl of middlebrook 7H9 broth containing rifampicin at 2µg/ml concentration (drug control). The test cryo vial was added with 350µl of middlebrook 7H9 broth and 50µl of partially purified bacteriocin (10mg/ml). About 100µl of *M. tuberculosis* H37Rv suspension (Mcfarland Unit 2) was added to all the vials and incubated at 37˚C. After 72 hours of incubation, 40µl of 0.1M $CaCl_2$ and 50µl of mycobacteriophage (phAE202) were added to all the vials and incubated for 4 hours at 37˚C. Then 100µl of the cell-phage mixture from each vial was added with 100µl of D-Luciferin and Relative Light Units (RLU) was measured using Luminometer (model: Lumat^3 LB 9508, make: Berthold). The inhibitory activity against *M. tuberculosis* was calculated based on the formula: Percentage RLU reduction = Control RLU– Test RLU / Control RLU × 100. The test RLU reduction by 50% or more when compared to control RLU was considered as positive for anti-tubercular activity.

RESULTS AND DISCUSSION

In the present study, *Pediococcus sp.* (2) was isolated from *Centropristis striata* and *Teuthida fish* intestinal tract. *Lactobacillus sp.* (2) were isolated from *Tilapia*. *Aerococcus sp.* and *Streptococcus spp.* were isolated from the gut of *Rastrelliger kanagurta* (Table 1a; Table 1b). The antibacterial and antimycobacterial activity of treated CFS samples are summarized in Table 2. CFS of five LAB isolates viz. BFO21, BFO22, BFO27, BFO29, BFO30 showed activity against *B. cereus* alone whereas BFO28 isolate have not shown inhibitory activity against any of the tested organisms. Plantaricin LPL-1 produced by *L. plantarum* have showed significant antibacterial activity against *S. aureus, L. monocytogenes, B. pumilus, B. amyloliquefaciens, E. faecalis* (Wang et al., 2018). In our study, BFO21 (*Lactobacillus sp.*) alone showed inhibition against *M. smegmatis* mc2155 by agar well diffusion assay indicating its antimycobacterial potential. Comparative 16S rRNA gene sequence analysis confirmed that *Lactobacillus spp.* BFO21 displayed 99.86% homology with *Lactobacillus plantarum* (GenBank accession number: MN367969). Silva et al., (2014) revealed that *L. plantarum* is one of the most important and versatile species and has many applications in various industries including pharmaceutical industries (Bravo et al., 2019).

The isolated *Lactobacillus plantarum* BFO21 was chosen for further purification of bacteriocin. Chloroform solvent extraction method was applied to purify the bacteriocin and the resultant lyophilized bacteriocin was screened against *M. tuberculosis* H37Rv using LRP assay. Bacteriocin of *Lactobacillus plantarum* BFO21 has shown reduction in RLU values by 98.00% at concentration of 35.53µg/ml. The result was compared with known anti-TB drug rifampicin at 2µg/ml that showed 91.93% of RLU reduction (Table 3). Previous studies have found that lactobacilli isolated from badger feces, wild boar feces or fermented milk products exhibited antimycobacterial activity against BCG and *M. bovis* (Mariam, 2009; Macuamule et al., 2016; Stedman et al., 2018; Bravo et al., 2019). To the best of our knowledge, this is the first study that showed the partially purified bacteriocin from *Lactobacillus* of *Tilapia* fish intestinal tract showing anti-tubercular properties (Bravo et al., 2019).

CONCLUSION

The burden of tuberculosis was reported high in India and treatment requires 6-8 months for new cases and 18-24 months for MDR TB. Lactic acid bacteria (LAB) and their bacteriocins received significant attention in the past decade due to its Generally Recognized as Safe (GRAS) status. Hits with improved cell penetration and with activity against *M. tuberculosis* should be prioritized. Bacteriocins of LAB certainly possess sufficient potential to merit hope for future therapies for treating intracellular infections. In this study, the *Lactobacillus plantarum* BFO21 obtained from fish gut has shown to produce antibacterial and antimycobacterial bacteriocins. The in vitro LRP assay of partially purified bacteriocins have shown significant anti-tubercular activity against *M. tuberculosis* H37Rv. Further purification and characterization of bacteriocins should be done to explore the possibility to develop the same as lead anti-tubercular compounds.

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Compliance with Ethical Standards: There are no laboratory animals and human subjects involved. The lactic acid bacteria were isolated from the intestinal tract of fishes obtained from fish slaughter-house.

Conflict of interest: All the authors declare that they have no conflict of interest.

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The Status of Lean Six Sigma Application Within SMEs In the Kingdom of Saudi Arabia: A High Level Technological Tool for Quality Improvement

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ABSTRACT

The present study has contributed to assessing the current state of Lean Six Sigma (LSS) implementation in Small and Medium-sized Enterprises (SMEs) in the Kingdom of Saudi Arabia (KSA). The study has also identified the significant success factors, ascertaining the benefits obtained and barriers obstructing the application of LSS in SMEs in KSA. The researchers were able to collect a total of 323 valid responses from the participants of the study without any missing from 2019 until 2020, using quantitative questionnaire which was distributed to a random sample to achieve the study's objectives. The findings of the survey highlighted that the most important critical success factors in implementing the LSS in SMEs are the availability of useful tools for communication and information, and a high level of awareness of the importance of LSS implementation in SMEs among the managers and employees. The results also showed that the essential benefits of applying LSS are that its application, this will reduce wastage and increase employee engagement and satisfaction. The lack of knowledge concerning LSS represented the most effective barrier hindering the implementation of LSS in SMEs in KSA. In conclusion based on the study's findings, Saudi SMEs must conduct workshops to spread knowledge concerning the effectiveness of LSS. Also, SMEs need to set a clear vision and objectives to ensure the successful implementation of LSS. This study can be of benefit to academicians and researchers as it adds to the technical areas of LSS knowledge, SMEs managers and directors and to the decision-makers in both governmental and private sectors by offering great insight into the significance of implementing LSS in SMEs for their financial growth..

KEY WORDS: LEAN SIX SIGMA, SMALL AND MEDIUM-SIZED ENTERPRISES, KINGDOM OF SAUDI ARABIA.

INTRODUCTION

In today's world, technology and research based businesses have become increasingly competitive which force companies in all industries to work efficiently to ensure sustainability, secure continuity, and profitability through the implementation of different research methodologies (Flor Vallejo, 2020). Many modern industries implement Lean Six Sigma (LSS) approach to solve several quality-related problems in many of their processes and for dramatically improving the quality of services, products, and processes (Antony et al., 2012; Gupta et al. 2019). Albliwi (2016) stated that "LSS is a business improvement method aiming to reduce stockholder value by improving quality, speed, customer satisfaction, and cost". It accomplishes this through merging tools and principles from Lean and Six Sigma. Motorola was the first to start using

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this method to express its quality program, and many international companies such as General Electric, Sony, Ford, Polaroid, and others have since proven successful in saving millions of dollars as a result of the correct implementation of the strategy Timans et al. (2016). In addition, there is a strong association between Six Sigma and the improvement of SMEs' performance, operational efficiency, and improved product quality (Vinodh et al., 2012; Singh and Singh, 2020).

SMEs play a vital role in national economies around the world, generating employment, value-added, and participating in innovation. For these reasons, SMEs are central to the efforts needed to achieve inclusive growth. Furthermore, Tripathi (2019) added that SMEs play an essential role in the modern economy as they represent the backbone of any country attempting to flourish and develop its economy. Besides, SMEs contribute about 60% of employment and 40% of national income in nearly all countries. Organization for Economic Co-operation and Development (OECD) (2016) revealed that SMEs are key players in an economy and the broader eco-system of firms enabling them to adapt and thrive in a more open environment. They contribute more positively to the digital transformation, which is essential for boosting economic growth and delivering inclusive globalization. Generally, in all countries, at any level of development, SMEs have a crucial role to play in a country's sustainable development. The SMEs sustainably is essential to poverty reduction, job creation, and sustain economic growth, (Tripathi 2019).

Based on previous studies conducted in the field of LSS in many organizations in Saudi Arabia, the researchers found that, though the popularity of the LSS method has been shown over the years to offer many benefits for those organizations who have implemented it, LSS received less attention in Saudi Arabia than in other countries and many KSA industries hesitate to implement this approach due to a lack of concrete evidence from empirical research into the current uptake of LSS in many eastern countries. This study is important in recent time because it focus on SMEs which is one of the key objectives outlined in the document Saudi Vision 2030 which is "raising the level of SMEs' contribution of 20 percent of GDP to 35 percent by 2030" (Rafiki, 2020).

Accordingly, and in line with vision targets, SMEs need to boost the improvement and development of their performance by 40 % to reach this goal. Therefore, the purpose of this study is to bridge the gap in the studies and to critically evaluate the present status of LSS implementation in Saudi Arabian SMEs by: Assessing the current state of LSS implementation in SMEs in KSA, identifying the significant success factors for the application of LSS in SMEs in KSA, ascertaining the benefits obtained from the application of LSS in SMEs in KSA and identifying the barriers obstructing the application of LSS in SMEs in KSA. The main question derived for this study is: What is the current status of LSS implementation in SMEs in KSA? To accomplish the main study objectives, the researchers aim to concentrate on the following sub-questions: What are the significant success factors for the application of LSS in SMEs in KSA? What are the benefits obtained from the application of LSS in SMEs in KSA? and what are the common barriers obstructing the application of LSS in SMEs in KSA?

MATERIAL AND METHODS

The current study adopted a descriptive research design which aims to provide a picture of a situation as naturally happens (Burns and Grove, 2003). Whereas research design is "the blueprint for the research process" (Cooper and Schindler, 2014). It precisely describes how the researcher conducted the study in scientific terms. It develops how the sample will be selected, specifies the data collection instrument used by the researchers and the research procedures. In this study, a descriptive research method used direct explanation, analysis, and description of a particular phenomenon without interference from the researcher (Cooper and Schindler, 2014). This method assisted the researchers to recognize and explain the features of the study population and their relationships. This study also employed an inductive approach which work from the "bottom-up, using the participants' views to form broader themes and create a theory interconnecting the themes" (Creswell and Plano Clark, 2007).

This study employed a simple random sampling technique to select the study sample. A random sampling technique is "A method that gives all elements of a study population an equal chance of being sampled" Mugenda et al. (2012). The sample size comprises a group of respondents, representing a part of the target population who's selected to serve that population (Cooper and Schindler, 2014). Therefore, the sample in this study was chosen randomly out of all Saudi SMEs to collect primary data which is "original search where collected data is designed by the researchers specifically to answer the research questions". Cooper and Schindler (2014). The researchers were able to collect a total of 323 valid responses from the participants of the study without any missing from 2019 until 2020.

Data was collected through a survey questionnaire instrument that is "any written instruments that present respondents with a series of questions or statements to which they are to react either by writing out their answers or selecting from among existing answers" (Brown, 2001). Furthermore, a questionnaire is one of the most popular data gathering techniques in quantitative research. The survey is prevalent because it is comfortable to build, adaptable, and capable of collecting a vast amount of information immediately in such a way that it is quickly processable. Therefore, the researchers used questionnaires to collect primary data. The researchers used the software Statistical Package for Social Sciences (SPSS) version 24 to conduct statistical procedures for questionnaire data. The statistical analysis, including the descriptive and inferential statistical methods. The inferential statistics method includes the correlation

coefficient; the mean values and standard deviations (SD) were employed by the researchers to represent the sample responses for each statement or item. The findings of the study have been presented, utilizing statistics and diagrams.

RESULTS AND DISCUSSION

This section deals with finding answers to the main research questions regarding the status of LSS application within Saudi Arabia's SMEs. Results of question one, which stated: What are the significant success factors for the application of LSS in SMEs in KSA? The results in Table 1 present the participants' perceptions regarding the significant success factors for the application of LSS in SMEs in KSA; it is clear that the overall mean value reaches (2.85), with SD (1.02). This mean value indicated that most participants tend to stand at the neutral position of responsibility. This result suggests that some participants identify some success factors while others do not perceive it.To understand if there are significant success factors to the implementation of LSS in SMEs, the detailed analysis of participants' responses is performed as follows:

Table 1. The participants' perceptions regarding the significant success factors for the application of LSS in SMEs in KSA.

Factors		Mean	SD	Ranking
The company has a clear vision which supports quality methodologies such as Lean /Six Sigma/LSS	323	0.00	0.00	7
Employees' engagement is supportive for Lean /Six Sigma/LSS implementation	37	3.14	1.46	6
Top management support and involvement in Lean /Six Sigma/LSS projects	37	3.39	1.23	3
The availability of a recognition and reward system	37	3.19	1.41	5
The company has a clear strategic direction and planning	37	3.24	1.32	4
Managers and employees have a high level of awareness about Lean /Six Sigma/ LSS implementation	37	3.46	1.30	2
The company has an effective tool for communication of information	37	3.54	1.45	1
Quality of human capital	2.85	1.02		

The results in Table 1 reveal that about the critical success factor, "The company has an effective tool for communication of information," it is noticed that the overall mean value to participants' responses is (3.54) with SD (1.45). The results indicate that most participants agree that the company has a useful tool for the communication of information, which might contribute to the application of new methodology. While, when participants provide their perceptions concerning statement 6 ("managers and employees have high awareness about Lean /Six Sigma/LSS implementation"), the results reveal that the mean value to participants' responses is (3.46) with SD (1.30).

Table 2. The participants' perceptions regarding the benefits obtained from the application of LSS in SMEs in KSA.

	Factors	Mean	SD	Ranking
1	The application of Lean/Six Sigma/ LSS has increased profit and financial savings	3.05	1.41	10
2	The application of Lean/ Six Sigma/LSS has increased customer satisfaction	3.35	1.30	7
3	The application of Lean/ Six Sigma/LSS has reduced the cost of poor quality	3.27	1.26	9
4	The application of Lean /Six Sigma/LSS has improved the quality of product and service	3.43	1.32	6
5	The application of Lean /Six Sigma/LSS has the time cycle of product and service	3.43	1.26	5
6	The application of Lean /Six Sigma/LSS has reduced the cycle time	3.30	1.31	8
7	The application of Lean/ Six Sigma/LSS has reduced the wastage in the process	3.62	1.28	1
8	The application of Lean /Six Sigma/LSS has improved the effectiveness of internal communication	3.49	1.33	4
9	The application of Lean /Six Sigma/LSS has improved the key performance metrics	3.51	1.30	3
10	The application of Lean /Six Sigma/LSS has increased employee engagement and satisfaction	3.51	1.35	2
	Quality of Human Capital	3.40	1.16	

This results mean that most participants agree that managers and employees have high awareness about lean/Six Sigma/LSS implementation. Whereas, when participants of the study responded regarding statement 3 (”top management support and involvement in Lean/ Six Sigma/LSS projects”), the results show that the mean value is equal to (3.39) with SD (1.23), which indicates that most participants stand at the crossroads, that they neither agree nor disagree. Therefore, there is no clear indication that top management has a real plan to support the methodologies' application.

On the other hand, when respondents provided their opinions regarding statement 2 (”employees' engagement is supportive for Lean/Six Sigma/LSS implementation”), the results reveal that the mean value is (3.14) with SD (1.46). The results indicate that there is no clear-cut answer to whether an employee's engagement is supportive of the implementation of the methodologies under investigation. Finally, when participants showed their level of agreement with statement 1 (”the company has a clear vision which supports quality methodologies such as Lean /Six Sigma/LSS”), the results show that no one provides an answer to this question. Therefore, we conclude that all companies do not have a clear vision that supports quality methodologies implementation, such as Lean/Six Sigma/ LSS. Hence, we find that SMEs face various difficulties and problems implementing such technologies, including the lack of human resources who have the right level of knowledge and even the culture of implementing quality improvement methods that are not available for most companies.

Results of question two, which stated: "what are the benefits obtained from the application of LSS in SMEs in KSA?"

The results in Table 2 show the participants perceptions regarding the benefits of the application of LSS in SMEs in KSA. The overall mean value is (3.40) with SD (1.16). This mean value indicates that there are various benefits of the application of the methodologies for the SMEs. Concerning participants' responses towards statement 7, (”The application of Lean/Six Sigma/LSS has reduced waste in the process”). The overall mean value to participants' responses is reaching (3.62) with SD (1.28). Thus, we conclude that most participants agree that the application of Lean Six Sigma/ LSS represents the most significant benefit and has contributed positively to reducing the wastage in the operational process. Reducing waste in any operational process is among the first requirements of applying quality standards.

While, when participants provide their responses about statement 10 (”the application of Lean/Six Sigma/LSS has increased employee engagement and satisfaction”), the results found that the mean value to participants' responses is (3.51) with SD (1.35). Therefore, we conclude that most participants agree that one of the benefits of applying quality methodologies is that it has increased employees' engagement and satisfaction. In addition to that, when respondents reported their views regarding statement 9 (”the application of Lean/Six Sigma/ LSS has improved the key performance metrics”), the results reveal that the overall mean to participants' perceptions is (3.51) with SD (1.30). Hence, we conclude that overall, participants agree that one of the benefits of the application of Lean/Six Sigma/LSS is that it improved the critical performance metrics. On the other hand, when participants of the current study, provided their perceptions regarding statement 3 (”the application of Lean/Six Sigma/LSS has reduced the cost of poor quality”), the results reveal that the overall mean value is (3.27) with SD (1.26).

The result indicates that participants neither agree nor disagree that the application has reduced the cost of poor quality. The variations among participants' responses might be related to the lack of professional use of such methodologies in SMEs. Finally, when participants reported their views to statement 1 (”the application of Lean/Six Sigma/LSS has increased profits and financial savings”), it is clear that the mean value is (3.05) with SD (1.41). This mean value indicates a high level of variation among participants regarding whether or not the application of such methodologies has increased the profit and financial saving in their companies.

Research of question three, which stated: "what are the common barriers obstructing the application of LSS in SMEs in KSA?"

Table 3. The participants' perceptions regarding the common barriers/challenges that obstruct the application of LSS in SMEs in KSA.

	Factors		Mean	SD	Ranking
1	Lack of Lean Six Sigma knowledge among the staff members	37	3.05	1.30	4
2	Lack of appropriate resources prevents the application of Lean/Six Sigma/LSS	37	2.95	1.22	6
3	Resistance to change among the employees	37	3.24	1.26	2
4	Organizational culture hinders the application of Lean/Six Sigma/LSS	37	2.97	1.26	5
5	Leadership style from top executive limits the application of Lean/Six Sigma/LSS	35	3.09	1.29	3
6	The complexity of using of Lean /Six Sigma/ LSS tools and techniques	37	2.92	1.21	7
7	Absence of business strategy limits the application of Lean/ Six Sigma/LSS	37	3.27	1.30	1
	Quality of Human Capital		3.10	1.05	

The results in Table 3 illustrate the participants' perceptions regarding the common barriers or challenges that obstruct the application of LSS in SMEs in KSA. The overall mean value is (3.10), with SD (1.05). This mean value generally indicates that there is a moderate level of barriers that obstruct the application of LSS in SME organizations. The results in Table 3 reveal that concerning participants' perception towards statement 7 ("Absence of business strategy limits the application of Lean/Six Sigma/LSS"), the results show that the overall mean value is (3.27) with SD (1.30). The result indicates that most participants agree that the absence of a business strategy limits the application moderately.

Whereas, when participants provide their perceptions concerning statement 3 ("resistance to change among the employees"), the results reveal that the mean value to participants' responses reaches (3.24) with SD (1.26), meaning illustrating that there is a moderate resistance to change among the employees towards the application of LSS in SMEs. On the other hand, the response toward statement 2 ("Lack of appropriate resources prevents the application of Lean/Six Sigma/LSS"), the results found that the mean value to participants' responses is (2.95) with SD (1.22). This result shows there is a high level of variation among the participants regarding this statement. Finally, when participants of the study report their perceptions regarding statement 6 ("The complexity of using of Lean/Six Sigma/LSS tools and techniques"), the results show that the mean value is equal to (2.92) with SD (1.22). This result indicates that the complexity of using such tools and techniques is not a critical barrier to obstruct the application.

The results of the study show that nearly all SMEs in KSA, represented by the SMEs who participated in this study, have never applied LSS (88.5%). Furthermore, even the 11.5% of the SMEs in this study that had used LSS were unaware of the importance of LSS application. Hence, the absence of the implementation of LSS was strongly related to a lack of knowledge regarding the LSS principles, and the concept is new to most of them. Other factors contributed to the lack of application of LSS in Saudi SMEs, such as the non-existence of a quality department and lack of LSS related training Tyagi, (2019). Other reasons can be as found by Fonseca and Da Fonseca, (2017) who found that SMEs managers believes that ISO 9001 standards and some tools from Lean toolkit can be sufficient for SMEs success whereas no need for LSS implementation. The study also showed that among the negative factors is a lack of clear vision in most Saudi SMEs regarding LSS implementation.

The results reveal that none of the SMEs, have a clear vision that supports quality improvement methodologies such as Lean, Six Sigma, and Lean Six Sigma (LSS). This finding are strongly support the study by Tyagi, (2019) in Indian SMEs that concluded SMEs lack of vision is one of the main reasons for not implementing quality improvement methods. On the other hand, it was found that other companies have a useful tool for the communication of information and that managers and employees have high awareness about Lean/Six Sigma/LSS implementation. It was also found that there are various benefits of the application of Lean/Six Sigma/LSS in the SMEs. Among the most important ones reported by participants are that the use of Lean/Six Sigma/LSS has reduced the waste in the process (Bhaskar, 2020) and has increased employee engagement and satisfaction (Bhaskar, 2020).

In addition, the use of LSS contributed to the improvement of critical performance metrics. This result confirms that the application of LSS has introduced various benefits (Tyagi, 2019).The standard critical success factors for SMEs in KSA to apply the LSS are having adequate tools for communication and information, and a high level of awareness regarding the importance of LSS among managers and employees. Therefore, the absence of most of the critical success factors of implementing LSS in SMEs in KSA prevents its adoption. In term of barriers the results show that there are no common barriers that obstruct the application of Lean/Six Sigma/LSS in SMEs. The result shows that most of the possible obstacles mentioned can be resolved if there is an actual adoption of the application of quality improvement methodologies. They were created to be implemented and incorporate certain principles and standards to be followed. Therefore, it can be an area for future research to investigate the reasons behind the lack of LSS implementation in SMEs as a methodology to improve their business operations.

Recommendations: Based on the findings of this study, the researcher recommends that companies should conduct workshops to spread knowledge concerning the effectiveness of LSS to increase productivity and improve the quality of outputs. Another recommendation is that Saudi SMEs need to set a clear vision and objectives for the successful implementation of LSS. The researchers also found that LSS training courses are necessary to build a sustainable environment for continuous growth and raise SME's contribution to the global market. Furthermore, SMEs should encourage their technical staff to obtain new knowledge about LSS, such as its principles and guidance. Lastly, both governmental and private sectors have to offer the required infrastructure to ensure the successful implementation of LSS in all Saudi SMEs.

This research contributes to many personal such as academic, the study added to the area of LSS knowledge, reducing it into individual work evidence based on theoretical perspectives and research possibilities that can help shape forthcoming investigations on various phases of production and sustainability. Also, SMEs managers and directors worldwide can consider the benefits of LSS adoption in their operations to become more competitive. Besides, the findings of this study will help the government, investors, and customers to classify the relevant factors that hinder the implementation of LSS in SMEs in KSA and work on to avoid them and address these factors critically. Furthermore, this study contributes to the decision-makers in both governmental

and private sectors: the findings of this study might assist them by offering great insight into the significance of implementing LSS in SMEs for their financial growth. This research has some limitations; for example, the researchers focused only in terms of SMEs in KSA and did not consider large companies. Therefore, the researchers suggest to conduct empirical studies capturing the implementation experience of LSS in large enterprises vs. SMEs another area for future research is a study on the obstacles and challenges facing the implementation of LSS in Saudi SMEs.

CONCLUSION

This study concludes that LSS is an approach that learns from past failures, one of which is too little support for management. Focus on clients, processes, workers characterize LSS as a way of building and developing a brand-new company culture and providing organizations with a tool for competitive advantage. LSS is one of the significant methodologies of quality management, seeking to increase productivity and improve the quality of method outputs. It emphasizes that imperfection is an opportunity for improvement. LSS is strongly connected with the establishment of businesses, and the combination of Lean and Six Sigma can bring dramatic improvements to SMEs. However, the finding of the current study indicated that almost all Saudi SMEs have never implemented LSS due to critical reasons such as lack of knowledge related to LSS and lack of quality improvement training related to performance.Besides, this study showed that many critical success factors could help Saudi SMEs implement LSS successfully; for instance, most Saudi SMEs in the survey have a useful tool for communication and information. Moreover, the managers and employees in these SMEs have a high level of awareness regarding the importance of LSS's role in company development.

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Influence of Physical Education and Sports on Social Cognition: an Analysis Based on Structural Equation Models

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ABSTRACT

Social factors are positively associated with social awareness related to physical activity. Participation in physical activity is linked to positive health outcomes and deliberate exercise in physical education and sports that improves mental health. This study presents a structural equation model (SEM) to evaluate the variables that most effect social awareness; empathy and compassion. The present study has used a representative sample of 32 students from a volleyball sport club of An Giang University, Vietnam National University Ho Chi Minh City, Vietnam. The sample was selected taking into account the 5% error rate and 95% confidence level. The results of the Kaiser-Meyer Olkin (KMO) global test and the Bartlett test show that factor analysis is complete, all works are statistically significant. Suitability tests show the model is suitable for the data. The findings of the present study conclude that, in all structures considered: interaction with authority figures, interaction with the opposite sex, evidence, confirming dissatisfaction, interaction with strangers and acting in public, the structure that most influences the underlying "empathy" is interaction with the opposite sex. The structure most likely to affect the underlying "empathy" is an assertion of discomfort.

KEY WORDS: SOCIAL COGNITION, SEM, PHYSICAL EDUCATION, SPORT, BRAIN, EXECUTIVE FUNCTIONS.

INTRODUCTION

This study presents the development of a structural equation model (SEM), which seeks to examine variables affecting social awareness (empathy and empathy) in An Giang university students, of the volleyball sport club. Five constructs were considered: Interplay with authority figures (TA), Interplay with the opposite sex (TB), be in evidence (TC), favored expression of discomfort (TD), interplay with strangers (TE) and act in public (TF) and two latent variables were used, empathy and sympathy. Social awareness is defined as the processes in which we draw inferences about the beliefs and intentions of others and how we consider social situational factors in making these inferences (Alvarez -Astorga et al, 2019).

Social awareness is impaired in a large number of neurological problems, including neurodegenerative diseases, neurological disorders and neurodevelopmental syndrome, and has become an important factor in differential diagnosis (Duclos et al 2018). Social awareness plays a role in teamwork and physical education in aspects like the player themselves because they have to evaluate what is going on, what they have to do to succeed and they must respond and adjust their playstyle based on teammates and rival teams (Koples, 2019).

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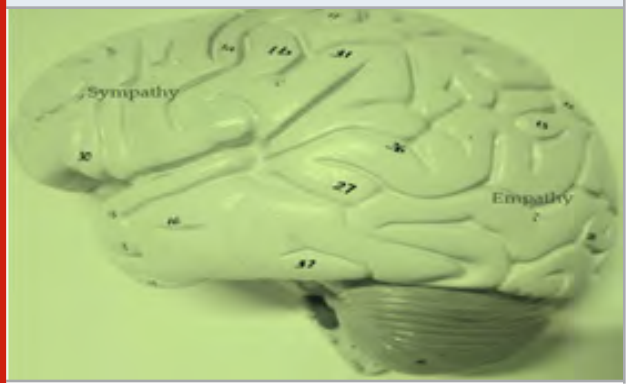
The remainder of the paper presents the general context of social awareness, the methods used and the analysis of results. Finally, the study concludes. Social cognition refers to the mental operations involved in understanding other people's thoughts and intentions, recognizing and perceiving emotions and understanding social interactions (Adolphs, 2001). Although social and non-social cognition share some overlapping operations (e.g. working memory, perception, etc.), some brain regions and networks have specifically been linked to processing social information (Green et al., 2015). Neural systems involved in processing social-affective stimuli, such as facial emotion and nonverbal social cues, include the amygdala, ventral striatum, ventromedial prefrontal cortex, anterior cingulate cortex and superior temporal regions (Adolphs, 2009). Higher level social cognition processes, such as inferring the intentions of others, are most commonly associated with activations in a broad' mentalizing network' including the medial frontal cortex, paracingulate and posterior cingulate cortex, temporal-parietal junction, superior temporal sulcus, and the temporal pole (Adolphs, 2009).

Social awareness is a concept introduced by neurologists, referring to a mental process that has been studied over the past few years in various clinical conditions such as schizophrenia and autism, increased attention deficit hyperactivity and antisocial personality disorder. It is defined as the ability of an entity to perform emotional processing, interpreting the intentions and beliefs of others in social situations (Christidi et al 2018). Neurosocial awareness is explained by the many neuronal connections of the cortical and cortical structures, with specific dominance of the frontal lobe. This concept studies the neurobiology of responses of empathy, sympathy, moral reasoning, recognition of the gaze and internalization of social rules, (Fede et al., 2016). Many physical education and sports, whether it be a team physical education and sportor an individual physical education and sport, include social cognition in multiple aspects. Studies have also shown that physical activity can increase and improve cognition in adults, which is one of the many benefits that playing physical education and sports can have, (Catalina et al, 2020).

The role of social cognition is present in individual physical education and sports competitions such as figure skating because the skater needs to pay attention to the program they are performing while also being sure not to fall on a jump and if they do, they need to adjust their performance to score higher on interpretation of music as well as knowing what the other competitors performed so they can try and score higher (Koples, 2019). The judgement of a physical education and sport can be biased and can impact the way the individual performs. If a team loses, the attitudes of players is most likely to have feelings of unfair judgements, on the other hand, the winning team would be more likely to feel they were judged fairly (Catalina et al, 2020). This puts an emphasis on the social aspect involving the referee or judge and how they determine what is right or wrong. Another aspect to consider while playing physical education and sports is the audience. It is also proven that physical activity leads to feelings of high self-confidence which can positively impact the individuals involved and if an individual is confident in what they are doing, they are more likely to perform well, opposed to having doubts in their abilities leading to a more negative outcome (Catalina et al, 2020).

Empathy and sympathy: Common sense is evoked by raising awareness and caring for others. The suffering of others by recognizing or responding to their suffering or needs. Sympathetic contexts appear to promote creative solutions, because people who empathize with others in suffering tend to seek new, desirable and prosperous solutions to alleviate suffering and promote happiness (Yang & Yang, 2016).Empathy, sharing and understanding the feelings of others, is a fundamental aspect of social capacity and a lack of empathy associated with aggressive behavior (Jolliffe & Farrington, 2004). Empathy is the ability to put yourself in another pair of shoes and really imagine how others must feel. Empathy is the ability to recognize and feel suffering or pity for others' suffering (Chapman, 2012). These abilities are even shown in specific parts of the brain. Sympathy is thought to use recognition functions in the frontal lobe of the third layer of the brain, while empathy is thought to include function in the lower right lobe of the brain (Chapman, 2012). Figure 1, shows the appearance of each lobe:

Figure 1: Sympathy and empathy regions



Sympathy can motivate a person to improve a situation, but it can cloud proper design judgment, and complicate relations with the person for whom you are researching and designing. Empathy, on the other hand, helps designers to increase their understanding while remaining objective (Chapman, 2012). Sympathy and empathy are different in another way as well. It is considered "easier" to feel sympathy than to feel empathy. Why is this? When we feel sympathy, we feel for another, but do not understand what the other person is truly feeling. When we are empathetic, we have built an understanding of another's emotions and feelings (Chapman, 2012).

MATERIAL AND METHODS

Structural equation modelling (SEM) is a class of multivariate models used for learning a causal

relationship among variables (exploratory modelling) or for testing whether the model is best fit by given data (confirmatory modelling). A general SEM includes the observed and latent variables, while their relationships are explained by a linear model whose parameters explain the cause or influence from one variable to another (Pruttiakaravanich & Songsiri, 2020). SEM has been widely used in behavioral research, such as in psychology, sociology, business and medical research (Price et al 2009).

Table 1. Social Abilities Questionnaire

Construct	Action	Variable
Interplay with authority figures (TA)	Write on the blackboard	T1
	Having to speak to a teacher	T2
	Ask me the teacher in class	T3
	Ask a question in class	T4
Interplay with the opposite sex (TB)	Start a conversation with the girl that i like	T5
	To tell a girl whom i like	T6
	something from her Give a kiss for the first time the girl that i like	T7
	Ask him to go out to the girl that i like	T8
Be in evidence (TC)	I make a joke in front of others	T9
	Make a fool of myself	T10
	in front of others I criticize	T11
	Stay without stuttering or	T12
	voice, the voice that I tremble to speak	
Favored expression of discomfort (TD)	Telling a friend that does not take my things without my permission	T13
	Tell a colleague who i did not like what he has said to me Tell a partner who does not	T14
	bother me when I am working To tell a partner that	T15
	is not always the center of attention	T16
Interplay with strangers (TE)	Being with other kids that don't know	T17
	Playing with a group of guys I know little	T18
	Ask for something to a colleague that almost don't know	T19
Act in public (TF)	Start talking with guys who don't know	T10
	Participate in a work of theater in the school	T21
	Singing in public	T22
	Dancing in front of everyone	T23
	Play an instrument in public	T24

Source: author's elaboration

We analyzed the relationships between six constructs (interaction with authority figures, interaction with the opposite sex, be in evidence, assertive expression of discomfort, interaction with strangers and act in public) and two latent variables (empathy and sympathy), which enables to analyze physical education and

sportsman's' attitudes in the social cognition. This was used as a database that met the responses from a survey of the,"The Social Abilities Questionnaire"(Caballo et al., 2012). The survey was applied to a representative sample of 32 volleyball players in a physical education and sport club in An Giang University, Vietnam. The sample was selected considering a margin of error of 5 % and a confidence level of 95 %. We used multivariate statistical techniques; regression and factor analysis in the statistical software SPSS 20.0

RESULTS AND DISCUSSION

Tables present the results of the Kaiser Meyer Olkin (KMO) test, any KMO is below 0.5, which is why it can be said that factor analysis is valid. The evidence of sphericity rejected at any level of significance considering the results of the Bartlett's sphericity test, the matrix of correlations is not an identity matrix. In the construction of the SEM model was used the builder tool of the statistical software SPSS 20.0. Was developed an analysis of main components of six constructs (interaction with authority figures, interaction with the opposite sex, be in evidence, assertive expression of discomfort, interaction with strangers and act in public).

Figure 2, presents the model developed: All signs of the slope coefficients are positive, showing a strong and direct correlation between the underlying variables and structures. Regarding the potential co-sensibility, the more influential structure is the interaction with the opposite sex, which has a coefficient of 0.99 (average). For its part, the structure that has the most effect on the empathy of the underlying variable is the assertive expression of discomfort, with a factor of 1.01 (average). There is a direct relationship between two latent variables (empathy and empathy), estimated with covariance 54, indicating that both variables are strongly correlated.

Table 3, presents the results of the goodness of fit test: Comparative Fit Index (CFI) and Tucker Lewis Index (TLI), which take values of 0.891 and 0.901 respectively, results that indicate a good fit. Finally, the Coefficient of determination was 0.86, is approaching 1 that indicates a good fit. The lower and upper limits of the statistic RMSEA are 0.051 and 0.253 respectively, which indicates that the setting is good. These results allow us to conclude that, the SEMs model developed is properly adjusted to data.

Table 2. Results of KMO and Bartlett's sphericity test

Construct	Variable	Measurement of sample adequacy of (KMO)	Approximate Chi square	Bartlett's sphericity Test	Sig
Interplay with authority figures (TA)	T1	.501	27.423	3	.000
	T2	.572	261.342	1	.000
	T3	.565	23.981	1	.001
	T4	.561	110.171	1	.000
Interplay with the opposite sex (TB)	T5	.549	119.509	3	.000
	T6	.531	121.465	1	.000
	T7	.538	120.691	1	.000
	T8	.528	32.231	1	.000
Be in evidence (TC)	T9	.500	34.223	3	.002
	T10	.592	111.302	1	.000
	T11	.665	223.781	1	.000
	T12	.541	23.130	1	.000
Favored expression of discomfort (TD)	T13	.543	23.509	3	.000
	T14	.581	25.465	1	.001
	T15	.522	127.691	1	.000
	T16	.528	26.233	1	.000
Interplay with strangers (TE)	T17	.511	112.423	3	.000
	T18	.651	25.425	1	.001
	T19	.502	27.691	1	.000
	T20	.525	116.233	1	.000
Act in public (TF)	T21	.661	32.443	3	.000
	T22	.581	115.415	1	.000
	T23	.542	227.601	1	.000
	T24	.573	136.203	1	.000
Source: author's elaboration					

Physical education and sport directly influences social cognition. The SEM developed in this work allows identifies the influence of the constructs; interaction with authority figures, interaction with the opposite sex, be in evidence, assertive expression of discomfort, interaction with strangers and act in public on the social cognition abilities. The model has identified a positive relationship and direct link between the six constructs and the two latent variables considered (empathy and sympathy). It has also identified a direct correlation between the two latent variables analyzed, for which an increase or decrease in any of them, will generate the same effect in the other.

Figure 2: SEM model diagram

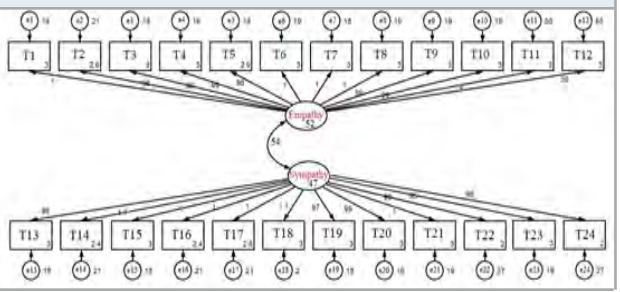


Table 3. Goodness-of-fit statistics of the estimated model

Fit statistic	Value	Description
Population error		
RMSEA	0.068	Root mean squared error of approximation
90 % CI, lower bound	0.051	
Upper bound	0.253	Probability RMSEA <= 0.05
p closed	0.061	
Information criteria		
AIC	14594.403	Akaike's information criterion
BIC	16774.232	Bayesian information criterion
Baseline comparison		
CFI	0.891	Comparative fit index
TLI	0.901	Tucker-Levis index
Size of residuals		
SRMR	0.07	Standardized root mean squared residual
CD	0.86	Coefficient of determination

CONCLUSION

The results of the Kaiser-Meyer Olkin (KMO) global test and the Bartlett test show that factor analysis is complete, all works are statistically significant. Suitability tests show the model is suitable for the data.

The findings of the present study conclude that, in all structures considered: interaction with authority figures, interaction with the opposite sex, evidence, confirming dissatisfaction, interaction with strangers and acting in public, the structure that most influences the underlying "empathy" is interaction with the opposite sex. The structure most likely to affect the underlying "empathy" is an assertion of discomfort.

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Quantitative Comparison of the Artifact of Six Cone-Beam Computed Tomography Systems in Endodontically Treated Teeth with Gutta Percha and AH26

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ABSTRACT

Artifacts are among the most important limitations of cone-beam computed tomography (CBCT). In different CBCT systems, the exposure options or the machine geometry might affect the diagnostic validity. This study aimed to compare the artifact rate in 6 CBCT systems in endodontically treated teeth with AH26. The effects of different voxel sizes on the artifacts in one of CBCT units was evaluated too.Twenty single-rooted teeth were randomly divided into 2 groups (n=10) and were instrumented up to the apical size of 25. The control group was left empty with no obturation, but case group were filled with gutta percha and AH26. Both groups were scanned by using 6 CBCT systems including NewTom VG, Planmeca, Kodak, Soredex, Vatech, NewTom Giano. CBCT scanning was performed via Vatech with 3 different voxel sizes (0.125, 0.2, and 0.3 mm^3.) OnDemand 3D software was used for analysis. Any deviation from the control group gray values was considered as artifact. The maximum, minimum, average and standard deviation of grey value in 4 points were measured. One-way ANOVA, independent t-test and Tukey's HSD post hoc test were used for statistical analyses of the data ($P<0.05$). Significant artifact was observed in Soredex, Planmeca, Kodak, and NewTom VG. While, NewTom Giano and Vatech showed no significant artifact. ($P<0.05$). artifact's presence was significant in images obtained with 0.3 mm3 voxel size, followed by 0.2 and 0.125 mm3, respectively. Different CBCT units have variations in artifacts. Even in units with fewer artifacts, it is critical to use a mode with small voxel size to reduce the artifacts.

KEY WORDS: ARTIFACT, CONE¬ BEAM COMPUTED TOMOGRAPHY, SEALER, VOXEL SIZE.

ARTICLE INFORMATION

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INTRODUCTION

The accuracy of cone-beam computed tomography (CBCT) has been investigated in several studies (May, et al 2013, Bernardes, R.A. et al. 2009, Kamburoglu, K., et al. 2009, Ozer, S.Y. 2010). The artifacts and noises sometimes interfere with clear vision of minor changes in CBCT images and consequently decrease the diagnostic accuracy of images. These artifacts are attributed to the high x-ray absorption by the objects of high density (Pauwels, et al. 2015) and results in two types of artefacts; 1) cupping artifact and 2) streaks and dark bands (White SC, 2014). When there is a radiopaque object in the canal, the produced artifacts negatively affect the diagnostic accuracy of details like vertical root fracture (VRF) (Kambungton, et al . 2012, Hassan, et al. 2010, Ozer, 2011). The effect of different materials on CT images has been investigated since 1970. Research revealed that different materials created different rates of artifact in images. Likewise, different rates of artifact have been reported in CBCT systems due to the several contributing factors (Wenzel, et al. 2005, Julia et al. 2004, Eliliwi et al. 2020).

There are various CBCT systems available for assessment of the dentofacial area. They differ in detector design, patient scanning settings, and data reconstruction parameters [Mozzo, et al.,1998, Kobayashi, et al. 2004, Araki, et al.,2004, Sukovic, 2003, Arai, et al., 1999, de Lima 2019]. The quality of CBCT images are significantly influenced by a number of scanning and reconstructing factors such as the field of view (FOV), voxel size, the number of basis projections used for reconstruction, and the image artifacts. CBCT systems are different in terms of image quality and ability of displaying the anatomic structure [Loubele, M., et al 2007, Loubele, M., et al., 2006, Loubele, M., et al.2008, Mischkowski, R.A., et al. 2008, Kwong, J.C., et al., 2008, Bryant, J.A., 2008, Liang, X., et al.].

The differences are more prominent and important in tiny anatomical structures such as periodontal ligaments and trabecular bone[Liang, X., et al.2010]. Several studies compared the diagnosis accuracy of various CBCT systems in VRF, or just compared a limited number of CBCT systems (Esmaeili et al.2012 , Safı et al.2015, Pauwels, et al 2013, Bamba, et al. 2013). Most of these studies evaluate the artifacts subjectively (Kamburoglu, et al. 2010, Hassan, et al.,2010, Ozer, 2011, Esmaeili et al. 20120) which can be influenced by the observer's situation. In order to overcome this limitation, the current study was conducted to quantitatively compare the artifact rate in six CBCT systems in endodontically-treated teeth with AH26 sealer and gutta percha and also the effect of voxel size on producing artifacts.

MATERIAL AND METHODS

This experimental study was performed on 20 single-rooted single-canal teeth. They were subjected to proximal radiography to confirm that the roots were quite healthy with no calcification, filling, or obvious fractures. The crowns were sectioned at the cementoenamel junction by using fissure diamond bur (D+Z; Germany). The samples were rinsed with NaOCl, and the length of the roots was adjusted to 13 mm. The sample teeth were randomly divided into case and control group (n=10 per group).The teeth preparations were performed by using Reciproc files on 1:6 reduction hand piece operated by a torque-controlled motor (VDW Silver reciproc motor). The files, speed, and torque were set according to the manufacturer's instruction. The R25 file with a tip size of 25 and a taper of 0.06 over the first 3 mm was used in a reciprocating, slow in- and out- pecking motion according to the manufacturer's instruction.

The samples in the study group were filled with AH26 sealer along with 0.04 tapered size 25 master gutta percha, followed by 0.02 taperd size 15 accessory gutta percha. The samples in the control group were left unfilled with no obturation. The upper surface of all canals were sealed with SE Bond Clearfil (Kuraray; NY, USA) and light-cured to prevent water penetration when placed in water during imaging. The samples in each group were mounted on a putty block at a minimum distance of 10 mm. The block dimensions were set according to the FOV adopted for imaging. The CBCT images of the samples were taken by using six CBCT systems including NewTom VG (QR SRL Company; Verona, Italy), Planmeca (Planmeca OY; Helsinki, Finland), Kodak (Trophy; Croissy-Beaubourg, France), Soredex (Soredex; Tuusula, Finland), Vatech (PaX-Flex3D; Vatech Co., Hwasung, Korea), and NewTom Giano (QR SRL Company; Verona, Italy). The CBCT images were taken with KvP and mAs set by operator of each device to gain the best quality with less effect of the two factors and the largest FOV (Table 1). By selecting the largest FOV, effect of the location on FOV has reduced.

Table 1. The characteristics of CBCT systems and imaging conditions

Imaging conditions / CBCT Systems	Kvp	mA	Time (ms)	FOV	Voxel size (mm)	Detector type
NewTom VG	110	3.9-5.6	3.5	20×25	0.125	FPD*
NewTom Giano	90	3	3.6	11×8	0.5	FPD
Vatech	85	5	4	10×8.5	0.2	FPD
Planmeca	80	8	5	10×8	0.4	FPD
Scanora	90	13	4	13×15	0.135	FPD
Kodak	85	5	4	17×13.5	0.2	CCD+

* Flat Panel Detector
\+ Charge-coupled device

The images were transferred to OnDemand software (Version 1.0; Cybermed Inc., 2010, UAS). As displayed in Table 1 and 2, four ROIs (Region of Interest) of 2-mm2 were selected in the mid axial plane (Hassan, et al. 2009). 1-mm out of the center of the teeth in

four directions (north, south, east, and west)(totally 40 samples in each group). The minimum, maximum, mean and standard deviation of the grey value was measured. The observations and calculations were done by an undergraduate dentistry student under the supervision of two experienced radiologists. Any deviation from the control group mean gray value was considered as artifact. After giving first stage results, Cone-beam computed tomography scanning by Vatech (as one the units with less artifacts in this study) was performed on automatic mode(85 kVp, 5 mA, and 0.6 seconds) with three voxel sizes of 0.125 mm3 and 5×5 cm FOV, 0.2 mm^3 and 5×9 cm FOV, and 0.3 mm3 and 14×9 cm FOV. Observation and evaluation was done as explained before.

The data were statistically analyzed by using SPSS software (version 16). Kolmogorov-Smirnov test was used to determine the normality of data. The descriptive features were measured including the central tendency and index of dispersion (mean and standard deviation). Analysis of variance of the groups was calculated regarding the studied features; the mean values of the groups were compared by using Duncan's test. The qualitative and ordinal parameters were analyzed by using nonparametric methods. Independent t-test was used to compare the case and control groups in terms of the mean rate of artifact. One-way ANOVA was used to compare the mean values among the six CBCT systems.

RESULTS AND DISCUSSION

Table 2 shows that the mean grey value was different among the six CBCT systems. The highest and lowest mean grey values were observed in images taken by Planmeca and Vatech systems, respectively. The independent t-test compared the mean grey values of the case and control group according to the imaging system (Table 2). The artifact value (The absolute numerical value of the mean grey value difference between the study group and the control group) in different systems was ordered as following, Scanora> Planmeca> NewTom VG> Kodak> Vatech> NewTom Giano. The difference was significant in Soredex, Planmeca, NewTom VG and KodaK ($P<0.05$) but insignificant in tow latter. Also the results revealed no significant difference in the grey value between case and control group with0.125 and 0.2 mm3 voxel sizes ($P>0.05$). But, at 0.3 mm3 voxel size, the grey value was significantly different ($P<0.05$); i.e., significantly more artifacts were observed at 0.3 voxel size.

Table 2. Comparison of the mean grey value and standard deviation (SD) in the case and control group according to the CBCT system

CBCT Systems	Case group Mean(SD)	Control group Mean(SD)	Difference between the case and control group
Planmeca	1707.5(519/72)	1177.9(221/69)	529.6
Scanora	1631.1(1314/88)	1075.6(162/79)	555.5
NewTom	1616.7(170/13)	1268.7(166/59)	348.08
NewTom Giano	1558.3(342/61)	1451.1(242/23)	107.02
Kodak	1419.9(403/89)	1144.1(271/03)	275.8
Vatech	949.8(637/76)	815.7(121/86)	134.1

The CBCT images can be negatively affected by the artifacts from the root filling materials, which decreases the accuracy, sensitivity, and specificity of this imaging technique in identifying the image details (White 2014, Khedmat, et al. 2012, Wanget al. 1998, Hassan, et al 2009). The current results revealed that despite the different mean grey value, the CBCT devices created different rates of artifact. Among the six studied systems, the highest and lowest artifacts were seen in Scanora and NewTom Giano, respectively. An artifact is any distortion or error in the image that is unrelated to the subject being study (White et al. 2014). Evaluation of gray value in the images as the presenter of X-ray attenuation pattern is a way to assess the non- uniformity and artifacts (Rabelo et al.2017). Artifact is sometimes due to the beam hardening phenomenon, in which the material absorbs more low-energy photons than the high-energy ones (Arai, et al 1999). This phenomenon creates two different types of artifact including cupping artifact and streaks and dark bands (White 2014). These lines lead to misdiagnosis of extra canals or VRFs, and false positive results(Araki, et al. 2004). The higher the atomic number of the material, the more artifacts would be observable (May et al.2013).

In several researches artifacts are studied subjectively (Kamburoglu, et al. 2010, Hassan, et al., 2010, Ozer, 2011, Esmaeili 2012) which can be affected by observing situation so in current study, artifacts were evaluated quantitatively by considering the difference between the numerical values of gray level in case and control group. Hassan et al. evaluated 80 extracted teeth placed in a dry mandible (Hassan, et al. 2009). Similar to some other studies, they found that the artifacts caused by the root filling such as sealer, gutta percha, metals, and silver cones drastically decreased the specificity of CBCT images; however, it did not affect the sensitivity (Kamburoglu, et al. 2010, Hassan, et al., 2009, Rabelo et al. 2017, Talwar, et al. 2016, Moudi et al. 2015 Shokri et al 2019).

Presencce of the sealer and gutta percha in the root canal reduce the specificity (Moudi et al. 2015). It is worth mentioning that the sealer per se causes more artifacts than the gutta percha per se (Decurcio et al. 2012). Since AH26 contains bismuth oxide, all the samples in the present study showed artifact and decreased the quality of CBCT images. Limited number of studies directily addressed the artifact in CBCT images, and most of them were focused on details such as root fractures and resorption (Hassan, et al. 2009, Melo, et al 2010, Karaçaylı, 2013, Patel, et al., 2013).The nature of artifacts is reported to be similar in different systems. Iikubo et al. compared 3D Accuitomo, Alphard VEGA, and CB Throne systems, and found no significant difference in the characteristics of the artifacts (Iikubo, et al. 2015). This similarity was attributed to the dependency of the artifacts on factors such as the position and size of the FOV and spatial resolution of the device. In any case, the quality of CBCT images is directly related to the imaging conditions.

The rate of artifacts and diagnosis accuracy of CBCT images are influenced by several factors such as the characteristics of the device and the imaging conditions. The device characteristics are the detector type, FOV, voxel size, and system-related artifacts. The imaging conditions are KVp, mA, position of the object in the FOV, and duration of radiation. The systems might also be different according to the amount of basis radiation for each image, data reconstruction parameters (algorithm), and device-related artifacts [8]. Accordingly, different studies employed different CBCT systems. Based on the detector technology, the general CBCT devices are divided into image intensifier tube/charged coupled device (IIT/CCD) and flat panel detectors (FPDs). Reports indicate that the IIT/CCD has increased pixel noise and higher image artifact than the FPDs, results in lower contrast and spatial resolution (Hassan, et al., 2010).

The FOV contributes to creating artifact as much as the detector does. Based on the adopted FOV, the CBCT devices are divided into three types of small, medium, and large. The FOV is directly related with the voxel size, and affects the spatial resolution and contrast. Larger FOV creates lower resolution and contrast, which directly influences the observation of anatomical structures (Hassan et al. 2010, Durack, et al. 2012, Kajan, 2012). The smaller the FOV, the higher the image quality and the lower artifact[Wang 1999, Costa, et al. 2012]. Regarding the direction of the object in the FOV, the more the distance between the object and centre of FOV, the more the radial-shaped artifacts (Lee, et al. 2002). Moudi et al. evaluated the effect of metal artifacts in different field of views, and found that in smaller FOVs, the sensitivity and specificity of the NewTom 5G system was 100%. In higher FOV, the sensitivity was decreased by 14% and specificity decreased by 11% (Moudi 2015). The present study investigated the general conditions of the systems; therefore, to create similar conditions, the highest FOV in each system was used which covered the object thoroughly. By using high number of samples and their distribution all over the FOV, and considering average of all samples, we aimed to reduced the effect of object position in the FOV. Yet, the present study reported high artifacts in Scanora (FOV= 15×13) or NewTom VG (FOV= 25×20), which could be due to the large FOV.

Voxel size is another factor that affects the quality of CBCT images [Muhammad, A.M.A. et al. 2020]. In this study there was no focus on FOV or voxel size in controlled condition, and interestingly different results were found; Planmeca and NewTom Giano units had nearly similar FOV and voxel sizes but showed completely different amount of artifacts. As several studies show the smallest voxel size increases the resolution and consequently the quality of images and diagnosis accuracy of VRF (Hassan, et al., 2010, Melo, et al. 2010, Iikubo, et al. 2015, Durack, C. et al. 2012). However, artifacts are still observed in small voxel sizes due to the presence of radiopaque materials such as gutta, sealer, or metal (Durack, et al. 2012). The present findings showed that the radiopaque content of AH26 sealer such as sulfate barium, bismuth oxide, and zinc oxide, caused this sealer to create artifacts on CBCT images in all three voxel sizes.

The present study used single software to eliminate the plausible effect of software in image reconstruction and consequently the artifact rate. Although Melo et al. studied four CBCT softwares and observed that presence of metal post in the canal significantly decreased the diagnosis accuracy in all the four softwares (Melo, et al. 2010). Hassan et al. compared five CBCT systems in detection of VRF at different voxel sizes. They concluded that iCAT at 0.25 mm3 voxel size showed the best diagnostic ability, followed by Scanora 3D at 0.2 mm3 voxel size, Accuitomo XYZ at 0.25 mm3 voxel size, and NewTom at 0.2 mm3 voxel size. The lowest quality was related to the Galileos at 0.3 mm3 voxel size. They attributed the differences among the results of different devices to factors such as the detector type, FOV, and voxel size (which affects the contrast and resolution), as well as the inherent artifacts of each system. However, Wenzel et al. reported that iCAT functioned better with 0.125 mm rather than 0.25 mm voxel size (Wenzel, et al. 2009).

Melo et al. evaluated the diagnostic ability of CBCT images in detecting the longitudinal root fractures in prosthetically-treated teeth. They observed better image quality and sensitivity in 0.2 than 0.3 mm3 voxel size (Melo, et al. 2010). Likewise, Ozer found that 0.2 mm3 voxel size was superior to 0.125, 0.3, and 0.4 voxel sizes because of its lower exposure and proper image properties (Ozer, 2011). Valizadeh et al. assessed the diagnostic accuracy of CBCT images in detection of VRF in presence of casting posts. They noted that 0.2 and 0.125 mm3 voxel sizes were not different in sensitivity, specificity, positive and negative predictive values. Therefore, 0.2 mm3 voxel size was recommended based on ALARA principle (Valizadeh, et al. 2015). Aligned with current study, Janqueira et al. reported no difference between 0.125 and 0.25 mm3 voxel sizes (Junqueira, et al. 2013). Whereas, Wenzel et al.

announced that Icat system functioned better in 0.125 mm3 voxel size than in 0.25 mm3 voxel size (Wenzel, et al. 2009). Comparing three voxel sizes, Kamburoglu et al. observed that 0.19 and 0.1 mm3 voxel sizes offered better quality than 0.3 mm3; however, the imaging took longer and the patient was exposed to higher radiation dose (Kamburoglu, et al. 2010). Similarly, Liedke et al. noted higher diagnostic quality at 0.2 and 0.3 mm3 voxel sizes compared with 0.4 mm3 voxel size in detecting external root resorption (Liedke, et al. 2009).

The present study announced the group with larger voxel size (0.3 mm3) to have the highest rate of artifacts, than the groups with smaller voxel sizes (0.2 and 0.125 mm3) with no significant artifact. This was in line with the studies carried out by Kamburoglu , Ozer and Melo (Kamburoglu et al 2010, Ozer 2011, Melo, et al. 2010). Artifacts were also observed in smaller voxel sizes due to the presence of radiopaque materials like gutta, sealer, or metal (Durack, et al. 2012). Presence of noise in small voxel sizes degrades the image quality (Karaçaylı, . 2013). It seems that more attention should be paid to the signal-to-noise ratio (SNR). Decreasing the voxel size would reduce the S/N in each voxel (Spin-Neto, 2013), which should be compensated with mAs and kVp; otherwise, it would reduce the image quality. Presumably, this can be the reason for the presence of artifact and low image quality in the group with 0.125 and 0.2 mm3 voxel sizes in the present study. Accordingly, considering ALARA, 0.2 mm^3 voxel size is suggested for more accurate evaluation of images and higher quality to investigate the details. Regarding the KVp, Esmaeili et al. evaluated the artifact caused by titanium implants and concluded that increasing the KVp resulted in decreased artifact in images (Esmaeili et al. 2012).

Having surveyed the imaging parameters, Jadu et al. concluded that the KVp was directly related with signal difference to noise ratio (SDNR) and adversely related with the mA (Jadu et al. 2011). Regarding the mAs, Scarfe pointed out that the ideal imaging parameters to reduce the artifact in CBCT was small FOV, small voxel size, short time, and low mA (Scarfe et al. 2009). However, Decurcio declared that decreasing the mAs resulted in increased artifact (Decurcio et al. 2012). In the present study, the most artifacts were created by Scanora and Planmeca, which had the highest mA. Withal, this factor was not directly and independently evaluated. Moreover, due to the proximity of the KVp values in all devices, except for the NewTom VG (110), no specific effect could be studied. Solutions have been suggested to reduce the artifact in images (Hassan, et al. 2009, Bechara et al. 2012), one of which is the use of softwares which can decrease the artifact caused by metals through reducing the beam hardening phenomenon (Bechara et al. 2012). However, the use of these methods is still controversial and requires extensive studies.

CONCLUSION

Within the limitations of this study it can be concluded that artifacts were observed in images taken by all the studied CBCT systems. Although units with less artifacts are suggesting. Besides that, artifacts were observed in all images with any voxel size, smaller voxel sizes were found to reduce the artifact. However, they are accompanied by more patient radiation dose. Hence, a balance should be considered in selection of voxel size.

Declaration: Authors declare that they have no conflict of interest. No identifying information about patients is included in the article.

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In situ Stress Assessment of the Impact of River Pollution in a Catfish, *Mystus tengara* Using Histopathological and Oxidative Biomarkers

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ABSTRACT

In the present study we have investigated various enzymatic (SOD, CAT and GST) and non-enzymatic antioxidant parameters (GSH and MDA/LPO) and histopathological biomarkers in the liver and kidney of *Mystus tengara* collected from the upstream and downstream of Chambal River. Results revealed that the activities of antioxidant enzymes like SOD, Catalase (CAT) and glutathione S-transferase (GST) activities were significantly higher in both the tissues of fish from downstream than from reference site ($p < 0.05$), demonstrating initiation of antioxidant defense mechanisms. Similarly, LPO levels (MDA) were elevated in both the tissues of fish from the downstream site than reference site while reduced glutathione (GSH) concentrations in both the tissues were significantly decreased in the fish of downstream ($P<0.05$). The histopathology of the liver of fish from downstream exhibited marked differences like vacuolization, hemorrhage, presence of glycogen granules, necrosis, dilation of sinusoids and congestion of blood vessels while kidney showed a reduction in Bowman's capsule space, degeneration of glomerulus, hemorrhage, necrosis vacuolation and reduction of tubular lumens. The histopathological changes were evidently associated with contamination, being more severe in kidney than the liver. The activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) were significantly increased in renal tissue than liver tissues which were clearly reflected also in histopathology. These results point out that wastewater from urban and neighbouring industries discharged into downstream of the river provoked the most significant oxidative stress in the native fish which was also reflected in histopathology. On the whole, the current study recommends that the biomarkers of oxidative stress along with histopathological studies can serve as a valuable tool for examining the adverse effects of wastewater effluents on fish..

KEY WORDS: BIOMARKERS, CHAMBAL RIVER, HISTOPATHOLOGY, IN SITU ASSESSMENT, OXIDATIVE STRESS.

ARTICLE INFORMATION

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INTRODUCTION

Freshwater environs are frequently used as dump yards of the industrial and urban wastes. Such anthropogenic activities severely affect the aquatic ecosystem and its biota. Anthropogenic activities are the main cause of aquatic pollution. Several organic and inorganic chemicals like plastics, pharmaceuticals, insecticides, and heavy metals have frightening impacts on freshwater ecosystems (Reddy 2016, Srivastava and Reddy 2019). Yet, our knowledge to predict their adverse effects correctly is still inadequate. Periodically, the organisms undergo for local adaptation or maladaptation upon the exposure to chronic pollution which could cause a high intraspecific unevenness of sensitivity among wild populations (Jacquin et al, 2020). Many pollutants, especially the heavy metals and xenobiotics present in wastewater are regarded as most hazardous due to their and persistent and non-degradable nature (Fatta-Kassinos et al, 2011, Kumar et al, 2019, Kumar et al, 2020). Many of these pollutants induce toxic effects through the production of reactive oxygen species (ROS) (Reddy 2017). The oxidative degradation of lipids (lipid peroxidation, LPO) is the widespread cause of reactive oxygen species (ROS) and therefore universally employed as a biomarker of fish health (Srivastava and Reddy 2017, Renuka et al, 2019, Brahma and Gupta 2020).

A number of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione S transferase (GST) and non-enzyme reduced glutathione (GSH) etc. are capable of protecting the tissues by stabilizing, neutralizing and or deactivating the effects of free radicals (oxidative damage). The role of these antioxidant enzymes against lots of toxicants was well studied in mammals (Kapoor et al 2010, Bhowmick et al 2015, Verma et al 2016, Singh et al 2018, Sutradhar et al, 2020) but still, such studies are comparatively scarce in fish species which can be effectively used as biomarkers for aquatic pollution (Javed et al, 2016, Srivastava and Reddy 2017, Louiz et al 2018, Corredor-Santamaría et al 2019, Tenji et al, 2020). It is well-known fact that the results of oxidative damage is directly associated with histopathology of the tissues (Alchalabi et al 2016, Wei et al 2019). Therefore, the studies on histopathological examination of target tissues along with the examinations of oxidative stress markers would provide an inclusive risk assessment and toxic potential of River pollution in fish and other organisms (Ratn et al 2018, Awasthi et al, 2019, Kucukler et al 2020).Hepatic, branchial and renal tissues of the fish are predominantly used as target organs for biomarker studies (Camargo and Martinez 2007, Kroon et al 2017, Mohamed et al 2020).

The liver and kidney of the fish is the major metabolic and vital excretory organ respectively. The failure of kidney function leads bioaccumulation of pollutants which cause major deformities in the renal tissue. Periodic examination of these biomarkers in fish may offer an estimation of both ecosystem and fish health. However, as pollutants are usually present as complex mixtures in the natural environment, the correct assessment and prediction of probable toxicity is a big challenge (Dévier et al 2011, Altenburger et al 2019, Kumari and Kumar 2020). Because of this complicatedness, the use of biomarker responses of an organism to a stressor as a tool at the individual, tissue, cellular, molecular levels is well established in ecotoxicological studies (Reddy 2012 a, b, Reddy and Baghel 2012, Reddy 2016, Srivastava and Reddy 2017, 2019).

Fish may ingest a cocktail of pollutants through the food chain, gills and skin. Fractions of certain chemical pollutants can accumulate in tissues and cause weakening of fish health. A number of pollutants in freshwater ecosystem could lead to oxidative stress in exposed populations. Our earlier studies performed in the Chambal River at Nagda (M.P.india) confirmed municipal and industrial pollution in this region (Reddy 2012 a, b, and 2017). Therefore, the present study is aimed to explore the association of oxidative stress and histopathological injuries induced by aquatic pollution of Chambal River in the liver and kidney of *Mystus tengara* which is commonly prevalent in this zone.

MATERIAL AND METHODS

Fish collection: The live adult tengara catfish, (*Mystus tengara*, Hamilton, 1822) irrespective of the sex and of similar size and weight (n=10), (8.3 ± 0.6cm; 7.2 ± 0.42g) were caught at two different zones (upstream and downstream) by means of a cast net with the help of skilled local fisherman, of the Chambal River at Nagda, Ujjain (23'27N and 75'25), (M.P.India) during winter months of 2018. The fish were placed in two separate containers (upstream and downstream) with river water and immediately transported to the laboratory for histopathological and oxidative stress examinations. Fish were washed and anaesthetized by 0.1 g/L of benzocaine and liver and kidney tissues were dissected out for the study of enzymatic and non-enzymatic antioxidants and histopathological studies.

Homogenate preparation: Both hepatic and renal tissues were taken out of the fish body. They were washed carefully with phosphate buffer and soaked. Afterwards, 10% of homogenate was prepared using homogenizing buffer (50 mM Tris -HCl mixed with 1.15% KCl at pH 7.4) by using a Teflon tissue homogenizer (Remi, India). The tissue homogenate was centrifuged (Refrigerated centrifuge Remi, India) at the 10,000 rpm for 20 min at -40C and the collected supernatant was directly stored in aliquots at -20^{0}C in glass vials for further analysis.

Oxidative stress markers

Antioxidant enzymes

Superoxide dismutase (SOD): The activity of Superoxide dismutase (SOD) in both hepatic and renal tissue was determined by an indirect method given by Marklund and Marklund (1974) with slight modifications. The technique is based on the ability of superoxide dismutase (SOD) to inhibit the autoxidation of pyrogallol into a yellow solution. The absorbance can be measured at 420 nm and expressed as µ/mg protein.

Catalase: Catalase (CAT) is a universally known antioxidant enzyme that degrades the hydrogen peroxide (H2O2) into water and oxygen. The activity of CAT was calculated by monitoring the decline in absorbance of H2O2 at 240 nm and expressed as µmol/mg protein/ min (Aebi, 1984). Glutathione S-Transferase (GST): GST activity was measured by the procedure given by Pabst et al (1974) with few slight modifications. This method is based upon the ability of GST to conjugate 1-chloro-2, 4-dinitrobenzene (CDNB) to reduced glutathione. The absorbance of conjugation is determined at 340 nm and expressed as units /mg protein.

Non-enzymatic Antioxidants: Reduced glutathione (GSH): GSH is the common intracellular low-molecular-weight thiol. It involves metabolic defensive roles, including reduction of hydroperoxide, detoxification, and free radical scavenging (Reddy 2016). With few modifications, the levels of GSH were determined as per the protocol of Jollow et al. (1974) and the absorbance was measured at 412 nm. Lipid Peroxidation (LPO): Lipid Peroxidation in both hepatic and renal tissue homogenates was estimated by the method given by Buege and Aust (1978). It was determined by quantifying the formation of thiobarbituric acid reactive substances (TBARS) which enumerated as malondialdehyde (MDA) equivalents and the absorbance was measured spectrophotometrically at 530 nm.

Histopathology: Hepatic and renal tissues of downstream and reference site fishes were taken out and fixed in Bouin's fluid for 48h. Tissues were cleaned under running tap water and dehydrated in ascending grades of alcohol, cleared twice in xylene and finally embedded in paraffin wax and 7µ thick sections were made by using a rotatory microtome. Sections were cleared in xylene, hydrated in serial dilutions of alcohol, stained with haematoxylene and eosin and finally mounted with DPX. The stained micro sections were evaluated under a compound microscope (Olympus BX46) and photographed by using the Omax 8.0MP Digital USB Microscope Camera.

Statistical analysis: The results of the current investigation were expressed as mean and standard error (mean ± standard error mean) for all the parameters. The data were tested for the significance by employing the software of student 't'test in Microsoft Excel Windows 10 version.

RESULTS AND DISCUSSION

Water quality is vital as it plays a central role in regulating various metabolic and physiological processes. The results of our earlier publication evidently revealed that physicochemical properties of surface water at downstream of Chambal River at Nagda, Ujjain (M.P.India) exceed the standard limits of CPCB.

Enzymatic and Non- enzymatic Antioxidant parameters: The activities of enzymatic and non-antioxidant parameters in the liver and kidney of *Mystus tengara* from the Chambal River at Nagda are presented in Table 1. Results clearly reveal that the SOD, CAT and GST activities were significantly elevated in both hepatic and renal tissues of fish from the downstream ((P>0.05) but the percentage of increase was much higher in kidney than in the liver. The percentage increase of SOD, CAT and GST activities were 115.1% and 103.4%, 78.99% and 277.2% and 80.77% and 94.96% for liver and kidney respectively.

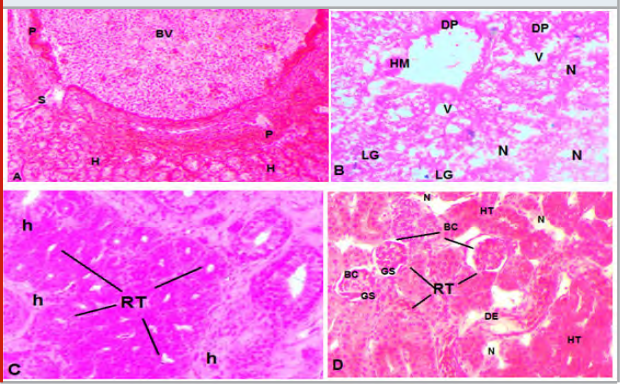
Table 1. Enzymatic and non-enzymatic activities in liver and kidney of *Mystus tengara*, Values are mean ±SE of six individual observations. Comparisons of means (upstream and downstream fish) were done by Student's t-test. * Significant at 5% level (p <0.05).

Parameter	LIVER			KIDNEY		
	Upstream	Downstream	%	Upstream	Downstream	%
Superoxide dismutase (SOD) U/mg protein	11.2 ±0.6	24.1± 3.3*	115.1	26.2 ± 3.1	53.3± 5.2*	103.45
Catalase (CAT) n mole/ min/mg protein	11.9 ±0.7	21.3 ±4.1*	78.99	2.2 ± 0.4	8.3± 0.8*	277.2
Glutathione-S-transferase (GST) µmole/ min/mg protein lipid peroxidation (MDA)	82.4 ±5.3	148.6±12.6*	80.77	53.6 ± 4.1	104.5 ± 9.7*	94.96
n mole/ hr/mg protein Reduced glutathione (GSH)	7.1± 0.31	29.3± 3.9*	312.6	4.1 ± 0.3	24.2 ± 2.2*	490.2
n mole/mg protein	158.6 ±9.8	97.2 ± 7.2*	-38.7	133.8 ± 9.7	53.3± 5.4*	-60.1

The lipid peroxidation levels (MDA) formation in both liver and kidney of *Mystus tengara* from upstream and downstream were displayed in Table 1. Results from Table 1 clearly reveal that MDA levels were significantly elevated in both the tissues of the fish from downstream of the River compared to that of control fishes from the reference site (upstream). The percentage of increase in MDA levels was significant (P<0.001) and were found as 312.6% and 490.2% in liver and kidney respectively. However, the levels of GSH in both the tissues (liver and kidney) significantly reduced compared to that of control (upstream). The percentage increase in GSH levels were 81%, and 83% respectively for liver and kidney.

Histopathological studies: The specifications of histopathological examinations in the liver and kidney of *M.tengara* from the reference site (upstream) and downstream site are shown in Figs. 1&2 respectively. Fish liver from reference site showed normal architecture (Fig1) with central veins, sinusoids, normal hepatocytes and glycogen granules. However, the micro sections of liver from downstream fish had shown several histopathological anomalies like necrosis, vacuolation, ruptured and congested central vein and few broken hepatocytes. Aggregation of Melanomacrophages (MMC) and a higher amount of glycogen granules was also seen (Fig1.B). The micro sections of the kidney of fish from upstream (control) exhibited normal structure with Bowman's capsule (BC), renal tubules (RT), epithelial cells (EC) glomeruli (G), proximal convoluted tubules (PCT), and distal convoluted tubules (DCT) with brush borders (BB) (Fig 3). However, the photomicrograph of renal tissue from downstream showed several pathological lesions including a reduction in Bowman's capsule space, degeneration of glomerulus, necrosis vacuolation and reduction of lumens (Fig 2.C&D).

Figure 1: A & B. Photomicrographs of the liver of *Mystus tengara* inhabiting in reference and polluted water. (A): Reference fish liver (B) Exposed fish liver; BV (blood vessel), H (hepatocyte), HM (hemorrhage) LG (lipid granule), P (pancreatic tissue), S (sinusoid), V (vacuolization). C &D. Photomicrographs of the kidney of *Mystus tengara* inhabiting in reference and polluted water. (C): Reference fish kidney (D) Exposed fish kidney; BC (Bowman's capsule), DE (degeneration of epithelium) GS (Glomerular shrinkage), N (necrotic cell), RT (renal tubule), H (haemopoeitic tissue), HT (hypertrophy) LG (lipid granule), P (pancreatic tissue), S (sinusoid), V (vacuolization). Sections were prepared from multiple fish liver and kidney tissues (four animals). All the sections were stained with haematoxylene and eosin and photomicrographs were taken using a light microscope with 400×magnification.



Earlier studies performed in the Chambal River at Nagda (M.P.india) confirmed municipal and industrial pollution in this region (Reddy and Renu Singh 2011, Reddy 2012 a, b, and 2017). The River Chambal at Nagda (M.P.India) is receiving approximately 18,500 to 19000 kl./day treated effluent from various industrial complexes and about 8000 Kl/day urban untreated wastewater at Juna Nagda area (downstream) which is found to be a major source of river pollution (Reddy and Baghel 2012, MPCB 2019). Fish species are so sensitive to changing the water quality and are subject to encounter with several types of chemical pollutants. Several researchers have conducted experiments to study the harmful effects of pollutants and many of those are linked it with the induction of oxidative and such studies will be useful to prevent or minimize the impacts of oxidative stress in animals stress (Reddy 2016, Srivastava and Reddy 2019).

In the present study, we estimated the impact of pollution on certain biomarkers of oxidative stress and histopathological biomarkers of aquatic pollution in a native catfish Mystus tengara from the Chambal River at Nagda (M.P.india). Several researchers reported that environmental pollutants could lead to the formation of excessive free radicals which cause oxidative stress and disturb cellular homeostasis in fishes (Lackner 1998, dos Santos Carvalho et al 2012, Yadav et al 2015). Oxidative stress markers like SOD, CAT, GST and GSH can serve as perceptive bioindicators of aquatic pollution in fishes (Javed et al 2016, 2017, Reddy 2016, Srivastava and Reddy 2017). SOD converts the superoxide radical anion (O.–2•) to H2O2. CAT counteracts or decomposes the toxic effects of H2O2. GST is the phase II type of detoxifying enzyme that protects the cellular macromolecules from ROS. Activities of enzymatic (SOD, CAT and GST) and non-enzymatic antioxidant parameters (LPO/MDA and GSH) were analyzed in hepatic and renal tissues to determine the impact of pollution on oxidative stress of liver and kidney of the fish and summarized in Table 1.

Results (Table 1.) clearly revealed a significant ($p<0.05$) and elevated levels of SOD, CAT, GST and reduced levels of GSH in the liver and kidney of the exposed fish compared to fish from upstream (reference site). The arrangement of SOD/CAT may be the earliest defense mechanism against ROS which are produced by the induction of pollutants. (Sharma et al 2012). The observed increase in SOD and CAT levels in liver and kidney of the fish exposed to wastewater specifies a strong detoxifying mechanism against the pollution-induced toxicity. We found similar interpretation in the hepatic tissue of the same fish, *Mystus tengara* exposed to wastewater in downstream of the river (Reddy 2016).

Similar results have also been documented by many workers in gill, liver and kidney of other fish species (Javed et al 2016, 2017, Reddy 2016, Srivastava and Reddy 2017, Tyor and Pahwa 2017, Ratn et al 2018, Kumar et al 2019, Chowdhury and Saikia 2020). Enhanced levels of CAT is frequently observed in various fish species frequently in the presence of ecological pollutants (Yadav et al 2015, Reddy 2016) as CAT in combination with superoxide dismutase SOD) stands for the first line of defence against oxidative stress (Ighodaro and Akinloye 2018). For that reason, increased levels of CAT in the current investigation reflect a tough and resistant antioxidant response generated by wastewater.

The elevated GST activities in liver and kidney of the *Mystus tengara* from the downstream of the Chambal River could be provoked to defend against the toxicity of pollutants. Similarly, the study of Samanta et al (2016) showed elevated levels of antioxidant enzymes like SOD, CAT and LPO levels in association with histopathological alterations in liver and kidney of a crucian carp Carassius auratus exposed to sewage water. In the same way, Samanta et al (2018) again confirmed increased activities of antioxidant enzymes along with histopathological anomalies in three fish species collected from different water streams of Korea. In another study, Chang et al (2019) showed that urban effluent can cause oxidative damage by increasing MDA content and antioxidant enzymes in the liver of *C. auratus*.

The recent study of Huang et al (2020) confirmed that pollutants of UV filters used in personal care products can cause oxidative damage by inducing ROS and alterations in SOD, GST, GSH, and MDA in the hepatic tissue of zebrafish. The study had shown the enhancement of SOD, CAT, and GPx activities, as well as the reduction in GSH content. But, in contrast, dos Santos Carvalho et al (2012) observed decreased SOD and increased GST in the liver of fish Tilapia (*Oreochromis niloticus*) from downstream of Monjolinho River (Brazil). The in situ assessment of Kim and Jung (2016) has shown a significant decrease in the enzyme activities of CAT, SOD, and GST in fish (*Z. platypus*) liver from the downstream. Apart from the antioxidant defence mechanisms, the reduced glutathione (GSH) (non-enzymatic) can also aid in the protection of the cell by scavenging of ROS/free radicals. Glutathione (GSH) is a tripeptide and serves as an important cofactor for antioxidant enzymes like GST and GPx. In the present experiment, GSH levels were significantly ($P< 0.05$) reduced in liver and kidney of exposed fish (Table 1). The reduction in GSH in hepatic and renal tissues of the exposed fish could be due to its oxidation to GSSG which occur during higher oxidative stress (Reddy 2016).

The induction of antioxidant enzymes takes place as a protection mechanism against the increased production of ROS. However, the responses of antioxidant parameters to pollution are not identical but differ for different species tissues and the amount of single or mixed pollutants. Great, variation can be found in wild situations (Livingstone 2001, Aljahdali and Alhassan 2020). The outcomes of this study suggest that the fish exploits both enzymatic and non-enzymatic mechanisms to abide by the effects ROS induced oxidative stress. For that reason, quantification of enzymatic and non-enzymatic (SOD, CAT, GST and GSH) constraints has been established as useful biomarkers of environmental pollution.Lipid peroxidation (LPO) is one of the major actions linked with cellular damage which expresses itself in the form of tissue injuries due to oxidative stress. Lipid peroxidation (LPO) changes the organization of cell membranes and affects the physiological functions of cell membranes (Reddy 2016, Srivastava and Reddy 2017).

In the present study, the MDA levels (lipid peroxidation product) were significantly ($P<0.05$) higher in both liver and kidney of *Mystus tengara* exposed to polluted water. The higher MDA levels potentially denote the damage of cell membranes. We found similar rationalization in the hepatic tissue of the same fish, *Mystus tengara* exposed to wastewater in downstream of the river (Reddy 2016). The elevated levels of LPO serve as a compensatory mechanism against surplus production of ROS/free radicals due to less effective antioxidant defence mechanism. The increased enzyme activities are indicative of the beginning of self-defence actions to alleviate impacts of ROS and free radicals to reinstate the redox balance and homeostasis in cells. The enhanced LPO and CAT, SOD, and GST in fish from downstream signify as combat mechanism against overproduction of ROS to minimize harm to the fish. The outcomes of current investigation evidently imply that the fish inhabiting downstream water were subjected to oxidative stress because of the high amount of pollutants and inadequate levels of antioxidants. Therefore, liver and kidney tissues were further processed for histopathological examinations.

A number of researchers applied histopathological signs as direct indicators of chemical exposures (Reddy and Rawat 2013, Javed et al 2016, Kumar et al 2017, Samanta et al 2016, 2018, Nofal 2019, Weber et al 2020). The liver is the chief metabolic organ and is the site for xenobiotics metabolism, detoxification and elimination of the toxicants. Exposure to xenobiotics induced histopathological changes in the hepatic tissue such as necrosis and hypertrophy. The apparent tissue damage in the liver and kidney might also be linked to the impact of oxidative stress. It is quite oblivious that the interactions of both ROS and toxicants interact with cellular apparatus cause tissue lesions and other forms of damage. The extensive accumulation of fats and portal swelling observed in the hepatic tissue reveals the detrimental effects of wastewater on native catfish. Accumulation of fat is a general cellular reaction to toxic pollutants that affects the lipid metabolism and weakens liver function. In such conditions, fish may be susceptible to parasitic infections due to reduced immunity (George et al 2017, Tan et al 2018).

Reduction in the immunity, enhanced secondary infections, impaired health in fish could ultimately result in a reduction in the reproduction process and an overall decline in the fish catch. The kidney is an important haemopoeitic and osmoregulatory organ in fish. It is also the site of excretion and transformation of xenobiotics. For this reason, entry of pollutants through the branchial artery may potentially induce histopathological alterations and alter the biochemical composition. Therefore, the expected pathological alterations in renal tissue can be used as biomarkers of environmental pollution.

The histopathological assessment of kidney demonstrated that fish exposed to pollution-induced the manifestations of degeneration of tubular epithelium, necrosis, hemorrhages, and shrinkage of the glomerulus and decreased the space between glomerulus and Bowman's capsule. Similar results were found in *Channa punctatus*

from sugar mill effluent at Aligarh, India (Javed et al, 2016), *Oreochromis mossambicus* from Bhima River of Maharashtra, India (Kumar N et al, 2017), *Carassius auratus* from Sincheon stream of Korea (Samanta, P et al, 2018), *Oreochromis niloticus* from Manzala fish farm of Egypt (Nofal, M.I 2019) and Hoplias intermedius and *Hypostomus affinis* from Doce River basin (Weber, A.A et al 2020).

CONCLUSION

This study has evidently shown that fish in downstream of the river live in the polluted environment was experienced from oxidative stress and tissue damage which consequently affects the fish growth, vulnerability to diseases and reproductive survival. The results in the current investigation clearly exhibit the threat posed by continuous discharge of treated effluents in the River may impair the fish health by inducing oxidative stress. The adverse effects of wastewater on the native and non-target catfish species is of concern as it may impair not only the fish health but also to the residents of the Nagda and neighbouring villagers who depend on the river for irrigation and for fish. The study recommends conducting more inclusive in situ measurements are required in order to resolve the current health status of fish populations in the Chambal River at Nagda (M. W.India).

Declaration of Competing Interest: None

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Assessment of Soil Nutrient Status of Priyadarshini Jurala Irrigation Project Command Area

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ABSTRACT

The Priyadarshini Jurala Project (PJP) is a multipurpose project across the river Krishna, located 18 km downstream of Telangana - Karnataka border (16020'15"E and 77042'15" N) on Krishna River in Telangana state falling in the Southern Plateau and Hill Agro-climatic region of India. Despite the importance of the irrigated area of the project, meager data is available on physico chemical properties and nutrient status PJP command area soils. To assess the soil physical and nutrient status of PJP command area, an investigation on soil fertility with respect to soil texture, pH, Texture, N, P_2O_5, and K_2O was conducted by using standard procedures. The texture of soils of Jurala command area varied from sandy loam to sandy clay loam. The mean pH values for kharif ranged from 4.81 to 8.14 and 4.96 to 8.12 with an average value of 6.45 and 6.80, respectively under PJP right and left main canal command area. The EC of the surface soil samples (0-30cm) indicated that the soils of Jurala command area are non saline. The mean value of available nitrogen was 184 kg N ha^{-1} in the PJP right main canal and 201 kg N ha^{-1} in the PJP left main canal. The available nitrogen is low (< 280 kg N ha^{-1}) in all soil samples. Out of the total samples collected in PJP right main canal, all the samples were high in available phosphorus whereas in the PJP left main canal 28, 25 and 47 per cent were low, medium and high in available phosphorus. In case of K_2O, 75, 23 and 2 per cent and 75, 19 and 6 per cent of the samples were low, medium and high in available potassium in PJP right and left main canal. For paddy crop under PJP command area, application of nitrogen and potassium is essential and the level phosphorus need to be reduced.

KEY WORDS: TEXTURE, PH, CEC, AVAILABLE NITROGEN, AVAILABLE PHOSPHORUS, AVAILABLE POTASSIUM.

INTRODUCTION

The varied geological, physiographic and vegetation characteristics resulted in the development of soil types (Reddy and Govardhan, 2012). Success of any production system is dependent on proper management of the physical and chemical properties of soils and the changes affect the availability of nutrients to crops by influencing the physical and chemical environment of the soil. The knowledge of the soils in respect to their characteristics and classification is important for optimizing land and input use for getting higher production from unit area. Periodical assessment of soil physical and chemical properties is an important aspect as it determines the manner in which it is being used for crop production. After provision of water from year through PJP project, the farmers are cultivating rice crop with soil manipulation and application of fertilizers which may alter the nutrient

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status and reaction of soil. The agricultural management impacts will vary the soil nutrients depending on climatic conditions (Ninijamerina, et al., 2017) and may also leads to changes in the properties of the soil (Qihua et al 2018).

The Priyadarshini Jurala Project is a multipurpose project across the river Krishna, located 18 km downstream of Telangana - Karnataka border (16020'15"E and 77042'15" N) on Krishna River in earst while Mahabubnagar district in Telangana state falling in the Southern Plateau and Hill Agro-climatic region of India. The Jurala irrigation project was mainly designed for growing ID crops in an area of 14262.8 ha under RMC and 32474.9 ha under LMC, (culturable command area of 41,360 ha) but farmers are growing paddy to a large extent in the command area. Periodical assessment of soil data base is limited for PJP irrigation command area. The present study deals with the assessment of soil pH, CEC, N, P_2O_5 and K_2O and soil texture of the command area of PJP project of Telangana in peninsular India as they help in soil fertility and productivity and in recommending proper nutrient application for higher yields without soil nutrient imbalances, which is required for judicious and scientific management of soil resources.

MATERIAL AND METHODS

Study area: The Priyadarshini Jurala Project is a multipurpose project across the river Krishna, near Revulapally village in Mahabubnagar district in Telangana falling in the Southern Plateau and Hill Agro-climatic region of India. It lies roughly between 16°-17° North Latitude and 77°-79° East Longitude. Mahabubnagar district is bounded by Karnataka state (West), Nalgonda district (East), Ranga Reddy district (North), and Kurnool district (South). The selected canals and distributaries at different locations and their salient features are presented below (Table 1).

Collection of soil samples: Initially preliminary survey of the command area was conducted by moving through the right (50 km) and left (100 km) main canals. This process helped to know the number of working distributaries, their length and ayacut under cultivation under each distributary. Using the preliminary information, 64 number of representative soil samples were collected under different distributaries with GPS location point during 2009 and 2010. The samples collected from the distributary numbers are D4, 5L, D34 under Right main canal (RMC) and D5, Ramanpadu left canal, D23 under Left main canal (LMC) (Table 1).

Table 1. Salient features of the distributaries selected for soil analysis under PJP right and Left main canal

Distributory No	Irrigation Potential (Ha)	Length of Canal (Km)	Offtaking Chainage (Km)	Localized Ayacut (ac)	Villages Benefited	Mandal
Right Main canal						
D4b	103.4	0.900	6.100	213	Rekulapally	Gadwal
5L	414.2	4.040	17.760	531	Chenugonipally, Venkampeta	Gadwal
D.34	3621.83	11.300	38.450	7549.3	Sasanool, Beerapuram, Kondair, Karupakula, Putandoddi, Munagala	Itikyala
Left Main canal						
D-5	94.62	1.100	10.950	254.1	Jurala, Atmakur	Atmakur
RLMC	1618.74	40.000	35.000	4000	Ajjakollu, Apparala, Ramakrishnapuram, Pampuram, Shakapuram, Ramapuram, Pebbair and Ragapur	Kothakota, Pebbair
D-23	1576.25	6.800	57.175	4112	Govardhanagiri, Ayyawaripally, Gumma dam, Thippaipalli	Veepangandla

The Physical and physico chemical properties of soil samples were analyzed for particle size analysis by Bouyoucos hydrometer method. The sand (2-0.05mm), silt (0.05-0.02mm) and clay (<0.02mm) fractions were calculated using hydrometer readings (Piper et al., 1966). The pH of soil was analyzed in 1:2.5 soil water suspension by using digital pH meter (Elico LI612 pH analyser) (Jackson, 1967) and the electrical conductivity was determined in the supernatant solution of 1: 2.5 soil water suspension as given by Jackson (1973) by using digital conductivity meter (Systronics Conductivity. – TDS meter 308µc).

The Chemical properties, available nitrogen were estimated by alkaline $KMnO_4$ method where organic matter in the soil is oxidized with hot alkaline $KMnO_4$ solution. The ammonia (NH3) evolved during the oxidation is distilled and trapped in boric acid mixed

indicator solution. The amount of NH3 trapped is estimated by titration with standard acid (Subbaiah and Asija, 1956) using Kelplus supra Lx, Pelicon equipment. The available phosphorus was extracted by employing Olsen's reagent (Olsen et al., 1954) and determined by Murphy and Riley method using ascorbic acid as reducing agent using spectrophotometer (Electronic corporation of India Ltd. V 5704). The available potassium was extracted with neutral normal ammonium acetate and was determined using Flame Photometer (Elico CL361) (Jackson, 1973).

RESULTS AND DISCUSSION

Success of any crop production is dependent on proper management of the physical and chemical properties of soils and those changes affect the availability of nutrients to crops. The texture of Jurala command area soils varied from sandy loam to sandy clay loam. The clay content ranged from 14 to 36 and 12 to 34 per cent, silt content ranged from 1 to 14 and 2 to 18 per cent and sand content ranged from 52 to 80 and 50 to 85 per cent in PJP right and left main canal respectively. The mean pH values for kharif 2009 and 2010 ranged from 4.81 to 8.14 and 4.96 to 8.12 with an average value of 6.45 and 6.80 under PJP right and left main canal command area. Out of the total samples collected, 46 and 25 per cent were moderately acidic (pH < 6.1) in nature, 50 and 65 per cent of the samples were slightly acidic to alkaline (pH = 6.1 to 7.8) and remaining samples were moderately alkaline (Table 2) in right and left main canal of Jurala command area (Table 2).

Table 2. Soil Physical and chemical properties of PriyadarshiniJurala Project right and left main canal

	pH (29.2°C)		EC (33.3°C)		N		P_2O5		K_2O		Texture		
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	% Clay	% Silt	% Sand
				Right main canal									
				Head Reach									
Range	7.75-4.74	7.73-4.88	0.392-0.103	0.296-0.078	213.2-163.1	188.16-150.53	294.2-100.7	299.6-146.2	126.3-47	142.5-87.4	28-14	12-4	80-61
Mean	6.00	5.96	0.25	0.19	334.49	320.57	190.21	212.825	77.775	116.6125	22.5	8.25	69.25
				Mid Reach									
Range	7.05-5.31	7-5.16	0.582-0.127	0.541-0.138	225.8-175.6	213.25-163.07	226.5-100.7	241-98.7	151.9-63.2	182.8-99.5	28-16	14-1	80-58
Mean	6.283	6.05	0.2303	0.256	194.44	188.159	165.91	153.06	86.97	139.51	23.4	8.8	67.8
				Tail Reach									
Range	8.41-5.86	7.87-4.83	0.655-0.136	0.687-0.104	213.2-175.6	188.16-150.53	258.7-65.7	272.3-56.9	306.4-56.4	450.2-86	36-20	14-6	73-52
Mean	6.871	6.816	0.261	0.2981	193.17	164.328	150.16	161.48	139.63	203.19	26.8	9.4	63.6
				Left main canal									
				Head Reach									
Range	7.86-4.56	6.96-4.37	1.09-0.205	0.655-0.128	280.5-213.2	225.79-137.98	130.-4-0	92.8-38.5	173.4-59.1	506.7-122.3	34-12	18-2	82-58
Mean	6.371	5.857	0.4835	0.2687	233.77	189.414	50.57	71.75	105.64	206.98	23.8	7.9	68.2
				Middle Reach									
Range	8.2-6.15	7.64-6.48	2.26-0.178	1.09-0.195	213.2-112.9	188.16-163.07	272.-3-0	288-34.1	392.4-67.2	235.2-103.5	32-14	11-3	78-57
Mean	7.49	6.91	0.74	0.65	191.29	174.05	156.40	169.80	170.85	151.54	22.88	7.63	69.38
				Tail Reach									
Range	8.84-5.71	8.22-5.32	1.14-0.183	0.699-0.156	263.4-150.5	225.79-163.07	251.-4-0	221.-5-0	258-56.4	264.8-103.5	34-12	18-2	85-50
Mean	7.44	6.56	0.52	0.36	217.42	189.55	59.47	75.52	114.84	158.46	22.22	8.78	69.00

In the head reach distributaries of right (D4) and left (D5) main canal, 88 and 70 per cent of the samples were acidic in reaction (< 6.5) and the percentage of samples showing acidity decreased towards the tail reach (Fig 1). The soils of the command area changed from acidic to neutral in nature from head reach to tail reach in the command area.

The pH value of a soil is influenced by the kinds of parent materials from which the soil was formed. Soils developed from basic rocks generally have higher pH values than those formed from acid rocks. Rainfall also affects soil pH. In the present study head reach distributaries are located in Gadwal and Atmakur mandals which received more rainfall than the other distributories. Water passing

through the soil leaches basic nutrients such as calcium and magnesium from the soil. They are replaced by acidic elements such as aluminum and iron. For this reason, soils formed under high rainfall conditions are more acidic than those formed under arid (dry) conditions. Some soils are acidic by nature and in other cases low pH is the result of prolonged and intensive fertilization and irrigation (http://www.savvygardener.com).

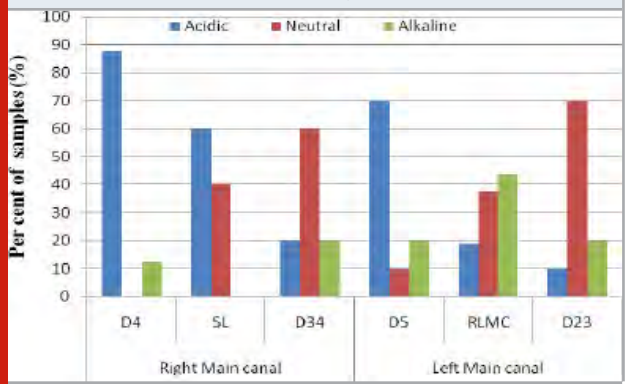
The EC of the surface soil samples (0-30 cm) indicated that the soils of Jurala command area are non saline. The EC ranged from 0.103 to 2.26 and 0.078 to 1.090 dS m^{-1} with a mean of 0.423 and 0.335 dS m^{-1} during kharif 2009 and 2010 respectively (Table 2). This was because of heavy rainfall during kharif 2010 which led to leaching of bases from the soil surface suggesting that the accumulation of low amount of soluble salts results in low EC. Rajeswar et al., (2009) measured that EC values for Agriculture Research Station, Garikapadu, Andhra Pradesh soils varied from 0.10 to 0.32 dS m^{-1} due to loss of bases (Sidhu et al., 1994) attributing to heavy rain fall.

The available nitrogen during kharif 2009 and 2010 ranged from 163 to 225 and 150 to 213.3 kg N ha^{-1} with a mean value of 193 and 177 kg N ha^{-1} in the PJP right main canal and it ranged from 113 to 280 and 138 to 225 N ha-1 with a mean value of 216 and 186 kg N ha^{-1} in the PJP left main canal respectively. The available nitrogen is low (< 280 kg N ha^{-1}) in all soil samples.In semi arid climatic condition, the available N status is commonly low and the soils are mostly coarse textured. Further, the organic matter addition to paddy soils is limited in the command area. The available nitrogen status of the study area low in all the samples as nitrogen being mobile in nature, the residual/ available N becomes poor in soils due to its losses through various mechanisms (Kumar, et al. 2014). Similar results were also reported by Yeledhalli et al. (2008) in sandy loam soils of Karnataka and Vasu et al. (2016) reported low available nitrogen in cotton growing soils of Mahabubnagar district, Sreeramsagar project and NSP left canal command area of Andhra Pradesh (Bhaskar Rao et al., 2002 and Rajeswar et al., 2009).

Table 3. Percent samples under particular pH range in PJP right and left main canal during kharif 2009 and 2010.

pH range	Right main canal			Left main canal		
	2009	2010	Mean	2009	2010	Mean
Moderately acidic (< 6.1)	39.3	35.7	37.5	19.4	30.6	25.0
Slightly acidic (6.1 to 6.5)	3.6	21.4	12.5	8.3	19.4	13.9
Neutral (6.6 to 7.3)	50.0	25.0	37.5	22.2	33.3	27.8
Slightly alkaline (7.4 to 7.8)	3.6	143	89	11.1	139	125
Moderately alkaline (7.9 to 8.4)	3.6	3.6	3.6	38.9	2.8	20.8

Figure 1: Percent of samples in a particular pH range in selected distributaries of PJP right and left main canal



The available phosphorus during kharif 2009 and 2010 ranged from 65 to 294 and 57 to 299 kg P_2O_5 ha^{-1} with a mean value of 166 and 170 kg P_2O_5 ha^{-1} in the PJP right main canal and it ranged from 24 to 272 and 20 to 288 kg P2O5 ha-1 with a mean value of 78 and 95 kg P_2O_5 ha^{-1} in the PJP left main canal respectively. Out of the total samples collected in PJP right main canal, all the samples were high in available phosphorus whereas in the PJP left main canal 28, 25 and 47 per cent were low, medium and high in available phosphorus respectively (Table 4). High available phosphorus in most of the soil samples under the command area was mainly due to indiscriminate use of P containing complex fertilizers like DAP, 17-17-17, 28-28-0 20:20:0, 19:19:19, 17:17:17, 12:32:18 or 14:28:28 by paddy growers of this region. For top dressing also, these complex fertilizers are used.

Analysis of large number of soil samples indicated that there is buildup of soil Phosphorus, especially in the top few cm of soil (Arévalo-Gardini, et al. 2015).Because of this, the response to applied phosphorus in paddy soils of PJP was low of not observed (Neelima et al., 2013) and also the submergence of rice field increases the availability of phosphorus. It has been reported that the change in soil Olsen P was positively linearly correlated with the P budget (P < 0.01) in the 0–20 cm soil layer (Arévalo-Gardini et al. 2015). A model of P fertilizer recommendation rate that integrates values of the change

in soil Olsen P in response to P budget need to be made for efficient use of P fertilizer (Qihua Wu et al. 2018).

Available potassium varied from 47 to 392 and 56 to 470 kg K_2O ha^{-1} with a mean value of 115 and 138 kg K_2O ha^{-1} during kharif 2009 and 2010. The available potassium ranged from 47 to 306 and 56 to 367 kg K_2O ha-1 with a mean value of 111 and 132 kg K_2O ha^{-1} in the PJP right main canal and it ranged from 56 to 392 and 67 to 470 kg K_2O ha^{-1} with a mean value of 124 and 149 kg K_2O ha^{-1} in the PJP left main canal during kharif 2009 and 2010. Out of the total samples collected; 75, 23 and 2 per cent and 65, 29 and 6 per cent of the samples were low, medium and high in available potassium in PJP right and left main canal (Table 4).

Table 4. Percent samples showing range of available phosphorus and potassium content in PJP right and left main canal

Available nutrient in soil	Right main canal			Left main canal		
	2009	2010	Mean	2009	2010	Mean
Phosphorus (kg P_2O_5 ha^{-1})						
Low (< 25)	-	-	-	42	6	28
Medium (25 to 59)	-	-	-	14	31	25
High (> 59)	100	100	100	44	64	47
Potassium (kg K_2O ha^{-1})						
Low (< 145)	86	64	75	78	53	65
Medium (145 to 340)	14	32	23	17	42	29
High (> 340)	0	4	2	6	6	6

Similar findings were observed by Kalyani et al. (2014) in Ranga Reddy district of Telangana state.Adequate (medium or high) available K in soils may be attributed to the prevalence of potassium-rich minerals like Illite and Feldspars (Sharma, et al., 2008). Potassium fixing capacity of soils is an important factor based on which K recommendations are given apart from available K status of soils. The K fixing capacity of soils depend on several factors like soil texture, type of clay minerals, potassium content in soils etc (Chaitanya et al. 2017) as these soils are sand and sandy loam in texture with low clay content. In general almost all the soils showed low potassium fixing capacity, generally the crop response to K application is expected, where the soils are low in available potassium. But, there are reports, where crop response to K fertilization is positive even in soils high in K status. Further, Potassium is low in soils with less organic matter and application of potassium fertilizers to the crop might have led to lower exchangeable potassium in soils (Chahal et al. 1976).

CONCLUSION

The texture of soils of Priyadarshini Jurala command area varied from sandy loam to sandy clay loam having an average value of 6.45 and 6.80, respectively under PJP right and left main canal command area and is non saline. The available nitrogen is low (< 280 kg N ha^{-1}) in all soil samples. In PJP right main canal, all the samples were high in available phosphorus whereas in the PJP left main canal 28, 25 and 47 per cent were low, medium and high in available phosphorus. The available potassium in 75 per cent of the samples was low. These results suggest that under Priyadarshini Jurala Project command, there is need to apply Nitrogen and potassium to meet the rice crop requirements and the level phosphorus need to be reduced.

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Comparative Assessment of Selected Indian Cultivars of Pigeonpea (*Cajanus cajan* L. Millsp) for *in vitro* Regeneration Using Apical Meristem Explants

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ABSTRACT

Development of effective regeneration protocol is a prerequisite for genetic transformation of pigeonpea owing to its recalcitrance behavior in tissue culture conditions. Screening of cultivars is considered to be one important factor for investigating the regeneration ability under in vitro conditions. Selected eleven Indian cultivars of pigeonpea were studied for multiple shoot bud induction and regeneration using apical meristem explants. The response of these cultivars under the influence of variable concentration of three different hormones namely 6-benzyl amino purine (BAP), kinetin (KIN) and thiadiazuron (TDZ) was investigated. BAP was found to be better compared to kinetin and TDZ for in vitro regeneration of these cultivars. It was observed that higher concentration of BAP was effective for multiple shoot bud induction and IPA-242 was promising revealing a maximum of 7 buds per explants at 3.0 mgL^{-1} of BAP. Similarly IPA-204 showed best response under the influence of different concentration of TDZ and a maximum of 10 buds per explants was observed at 0.30 mgL^{-1} of TDZ. The overall response of these cultivars under different concentration of kinetin was poor though IPA-2013 was found to be best with 4 buds per explants at 3.0 mgL^{-1} of kinetin. The rooting of the shoots derived from the apical meristem explants was found to be better when treated with 1- Naphthalene Acetic Acid (NAA) as compared to Indole-3 Acetic Acid (IAA) and Indole-3 Butyric Acid (IBA). Further it was observed that 0.2mgL^{-1} of NAA worked best for most of the cultivars for rooting as evident from number of primary roots. The screening of these cultivars of pigeonepea for in vitro regeneration ability exclusively from apical meristem explants has widened the scope of developing efficient regeneration and genetic transformation protocols.

KEY WORDS: APICAL MERISTEM, MULTIPLE SHOOT BUD INDUCTION, PIGEONPEA, HORMONES, REGENERATION.

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INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh]is an important protein rich grain legume predominately grown in Indian subcontinent, South East-Asia and East Africa, the genome of which has been sequenced (Singh et al. 2012 and Varshney et al. 2012). The crop productivity is hindered due to several constraints like limited genetic resources, low level of genetic diversity, plethora of biotic and abiotic stresses (Bohra et al. 2010). Conventional plant breeding, molecular breeding and genomic assisted breeding approaches are being used for legume crop improvement (Pratap et al. 2018; Bohra et al. 2020). The identification of genes associated with desirable agronomic traits in pigeonpea is comparatively easier due to the availability of genome sequence and could be used for transgenic production. Still the availability of efficient and reproducible in-vitro regeneration protocol is lacking in pigeonpea pea and other legumes in general as these are considered to be recalcitrant to *in-vitro* regeneration under tissue culture conditions (Chandra and Pantel 2003; Pratap et al. 2018).

Substantial efforts have been made to develop efficient *Agrobacterium*-mediated genetic transformation and transgenic pigeonpea production (Geetha et al. 1999, Lawrence and Koundal 2001, Satyavathi et al. 2003, Prasad et al. 2004, Surekha et al. 2005; Sharma et al.2006; Surekha et al. 2014; Ghosh et al. 2017; Karmakar et al. 2019). In pigeonpea *in-vitro* regeneration via organogenesis using different explants like leaf, cotyledons, cotyledonary nodes, embryonal axes, leaf petiole, embryo, embryonal axis attached cotyledons, auxillary buds and apical meristem among different cultivars has been extensively reviewed (Krishna et al. 2010 and Pawar et al. 2014). Leaf tissues were predominately used as explants source for in vitro regeneration of pigeonpea (Eapen and George 1993, Singh et al. 2002, Dayal et al. 2003, Kashyap et al. 2011, Asande et al. 2016, Abhijeeta and Rajesh, 2018).

Other explants source like cotyledons and cotyledonary nodes (Banala et al. 2016 and Jasani et al. 2017), embryonal axes (Raut et al. 2015), leaf petiole (Nalluri and Karri 2017), embryonal axis attached cotyledons (Karmakar et al. 2019) and auxiliary bud (Vijay Kumar et al. 2016; Kumar et al. 2016) have also been recently reported for in vitro regeneration of pigeonepea with different cultivars. There are only few reports of apical meristem as explants source for direct organogenesis (Kumar et al. 1984; Cheema and Bawa 1991; Franklin et al. 1998 and Parekh et al.2014) attempted with cultivars AL 15, ICP 6917, ICP 6974, ICP 7119, ICP 7263, Vamban, one wild and GT 102 (Karmakar et al. 2019).

Genotype dependent varying regeneration responses have been reported in pigeonpea using variable explants sources, though apical meristem has not been extensively studied. The screening of more cultivars for direct organogenesis exclusively for apical meristem explants needs to be attempted for evaluating the variability in the in vitro regeneration efficiency. Based on the literature survey an attempt has been made to evaluate eleven selected Indian cultivars of pigeonpea for multiple shoot bud induction and regeneration. The effects of variable concentration of growth regulators BAP, Kinetin and TDZ for multiple shoot bud formation among these cultivars were also assessed to reveal genotype dependent variability.

MATERIAL AND METHODS

The eleven cultivars of pigeonpea procured from ICAR-Indian Institute of Pulses Research, Kanpur were IPA-2013, IPA-3088, Pusa-9, IPA-34, IPA-204, IPA-242, T-7, IPA-61, IPA-337, IPA-341 and IPA-98-3 and were used insert in the present study. The seeds prior to germination were surface sterilized using 1% cetrimide solution, 70% ethanol and 0.2% HgCl2 as reported earlier (Kashyap et al. 2011; Kashyap et al. 2014) The apical meristem explants of approximately 1.0 cm size were excised aseptically from 10 day germinated seedlings. The standard MS culture medium (Murashige and Skoog 1962) with variable concentration of growth hormones BAP, Kinetin and TDZ was used for multiple shoot bud induction and regeneration studies.

The explants with or without shoot initials were sub cultured repeatedly after 15 days. Numbers of shoot buds were counted after 30 days of inoculation. For each experimental set up 10 explants were used with each concentration and experiment was repeated twice. After each successive subculture within 15 days, the well-developed shoots were rooted on MS media with different concentration of NAA, IAA and IBA. The explants with or without shoot initials were sub cultured repeatedly after 15 days. Numbers of shoot buds were counted after 30 days of inoculation. For each experimental set up 10 explants were used with each concentration and experiment was repeated twice. After each successive subculture within 15 days, the well-developed shoots were rooted on MS media with different concentration of NAA, IAA and IBA. The culture conditions of cool white fluorescent light at 25±2^{0}C with 16 hours light and 8 hour dark interval was maintained in plant tissue culture lab.

RESULTS AND DISCUSSION

Genetic transformation has immense potential for legume crop improvement but due to the lack of efficient regeneration methods, limited success has been achieved (Pratap et al. 2018). Plant regeneration through organogenesis has been preferred in pigeonpea genetic transformation and several efforts have been made to investigate the factors influencing *in-vitro* regeneration using different cultivars. *In-vitro* regeneration by organogenesis of pigeonpea has been attempted using diverse explants like leaf, cotyledons, cotyledonary nodes, embryonal axes, leaf petiole, embryo, epicotyls, embryonal axis attached cotyledons, auxiliary buds and apical meristem with more than fifty diverse cultivars (Krishna et al. 2010, Pawar et al. 2014 and Pratap et al.

2018). Several factors like genotype selection, explants tissues, media composition, and plant growth regulators substantially influence the plantlet regeneration via organogenesis in legumes that is amenable to efficient genetic transformation (Krishna et al. 2010, Pawar et al. 2014 and Pratap et al. 2018).

Screening of diverse genotypes or cultivars is considered to be the major factor for deciphering the inherent regeneration potential *via* organogenesis (Chandra Venkata et al. 2019; Bohra et al. 2020). More than fifty pigeonpea genotypes have been studied for *in vitro* regeneration both *via* organogenesis and somatic embryogenesis to reveal the inherent regeneration ability (Krishna et al. 2010). In the present study selected eleven Indian cultivars of pigeonopea were assessed for regeneration via organogenesis using apical meristem explants under influence of variable concentration of growth regulators namely BAP, Kinetin and TDZ as reported with leaf and plumule junction explants (Kashyap et al. 2011 and Kashyap et al. 2014).

These selected Indian cultivars of pigeonpea when subjected to variable concentration of BAP hormone ranging from 0.5-4.0 mgL^{-1} revealed variability in regeneration ability as evident from number of buds per explants as shown in Table-1. The cultivar IPA-242 showed best response with a maximum of 7 buds per explants in the presence of MS media supplemented with 3.0 mgL^{-1} BAP. The response of cultivars IPA-2013, IPA-2014 and IPA-61 was also comparatively better at higher concentration of BAP (Kashyap et al. 2014).

Overall higher concentration of BAP was found to be better for direct organogenesis as reported earlier irrespective of explants used (Krishna et al. 2010). The shoot bud induction for all the eleven cultivars with their best responsive concentration of BAP is shown in Figure-1(a-k). Mulitple shoot bud induction and regeneration exclusively in the presence of BAP has earlier been reported for cultivars ICP 6917, ICP6974, ICP 7119, ICP 7263 Vamban and one wild species (Kumar et al. 1984 and Franklin et al. 1998). A total of 12 numbers of maximum shoots has been reported from apical meristem explants in the presence of BAP (Franklin et al. 1998).

The response of these cultivars was also evaluated in the presence of different concentration of TDZ ranging from 0.05-0.4 mgL^{-1} (Table-2). The response of cultivar IPA-204 was found to be best with 0.30 mgL^{-1} of TDZ resulting in a maximum of 10 buds per explants. To the best of our knowledge there are no reports of in vitro multiple shoot bud induction and regeneration from apical meristem of pigeonpea in the presence of TDZ (Krishna et al. 2010).The concentration of TDZ in the range of 0.25-0.30 mgL^{-1} was found to be effective for shoot bud induction for these cultivars of pigeonpea. In case of cultivars IPA-242, IPA-337, IPA-341 and IPA-98-3 only single bud was observed irrespective of different concentration of TDZ used.

Table 1. Effect of BAP on multiple shoot bud induction using apical meristem explants (number of shoots / explant) for eleven cultivars of pigeon pea after 4 weeks of culture with an average of 10 replicates and means with different letters differ significantly at p=0.05.

BAP (mgL^{-1}) → Cultivars ↓	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
			Number of shoots (Mean ± S.D.)					
IPA-2013	1.7±0.4^{a}	1.7±0.4^{a}	3.3±0.6b	2.4±0.4^{a}	3.1±1.2^{b}	4.4±0.6^{ab}	4.4±1.3^{b}	3.9±1.5^{b}
IPA-3088	3.5±0.5^{b}	3.7±1.0^{b}	4.4±0.4b	5.9±3.0^{b}	5.3±0.6^{b}	3.9±0.9^{b}	4.3±1.1^{b}	4.7±2.7^{b}
Pusa-9	1.9±0.7^{a}	2.6±1.2^{a}	1.0±0.0a	3.3±0.7^{a}	1.0±0.0^{a}	1.3±0.4^{a}	3.5±1.5^{b}	4.7±0.4^{ab}
IPA-34	2.7±0.7^{b}	1.0±0.0^{a}	2.8±0.9b	1.0±0.0^{a}	2.2±0.4^{a}	3.0±0.0^{b}	3.8±0.6^{ab}	3.8±1.8^{b}
IPA-204	1.0±0.0^{a}	3.0±0.0^{b}	4.3±0.4b	3.5±0.5^{b}	1.5±0.5^{a}	4.6±1.9^{ab}	3.7±0.45^{b}	1.7±0.8^{b}
IPA-242	1.0±0.0^{a}	1.2±0.4^{a}	1.0±0.0a	1.9±0.3^{a}	1.9±1.1^{a}	6.2±0.6^{a}	1.4±0.7^{a}	3.7±0.4^{a}
T-7	1.0±0.0^{a}	1.4±0.9^{a}	1.4±0.8a	1.6±1.2^{a}	2.0±0.8^{a}	2.3±1.1^{a}	2.6±1.2^{b}	4.7±1.0^{ab}
IPA-61	1.0±0.0^{a}	1.0±0.0^{a}	5.7±0.9ab	1.0±0.0^{a}	3.2±2.0^{b}	4.6±0.9^{b}	4.9±1.4^{b}	3.8±0.6^{a}
IPA-337	1.0±0.0^{a}	1.0±0.0^{a}	3.4±0.6ab	1.0±0.0^{a}	3.1±0.3^{b}	1.0±0.0^{a}	1.1±0.3^{a}	1.0±0.0^{a}
IPA-341	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
IPA-98-3	1.0±0.0^{a}	3.5±0.5^{b}	1.0±0.0a	1.0±0.0^{a}	3.3±0.4^{b}	1.0±0.0^{a}	3.2±0.4^{b}	4.1±0.6^{ab}

Similarly when these cultivars were subjected to different concentration of kinetin ranging from 0.5-4.0 mgL^{-1}, they showed variability in terms of multiple shoot bud induction and cultivar IPA-2013 showed best response with a maximum of 5 buds per explants with 3.0 mgL^{-1} kinetin. It was also observed that many of the cultivars like IPA-204, IPA-242, T7, IPA-61, IPA-337, IPA-341 and IPA-98-3 showed no response for multiple shoot bud induction under different concentration of kinetin. In general, higher concentration of kinetin was found to be effective for shoot bud induction for most of the cultivars. Similar studies has been perfomed with cultivar AL-15subjected to different concentration of kinetin ranging from 0.1- 9.0 mgL^{-1}. The lower concentration in

the range of 0.5-3.0 mgL^{-1} was found to be better resulting in healthy shoots while higher concentration resulted in the formation of clustersalong with BAP (Cheema and Bawa 1991). Among these three hormones tested, BAP was found to be comparatively better as compared to kinetin and TDZ for in vitro multiple shoot bud induction and regeneration as reported earlier (Kumar et al. 1984, Cheema and Bawa 1991 and Franklin et al. 1998).

Figure 1: Multiple shoot bud induction from apical meristem explants of different cultivars of pigeonpea showing their best response in MS media supplemented with variable concentration of BAP (in mgL^{-1}). (a)IPA-2013 (3.0), (b)IPA-3088 (2.0), (c)Pusa-9 (4.0), (d)IPA-34 (3.5), (e)IPA-204(3.0), (f)IPA-242(3.0), (g)T-7 (4.0), (h) IPA-61(1.5), (i)IPA-337 (1.5), (j)IPA-341 (1.0), (k)IPA-98-3 (4.0).

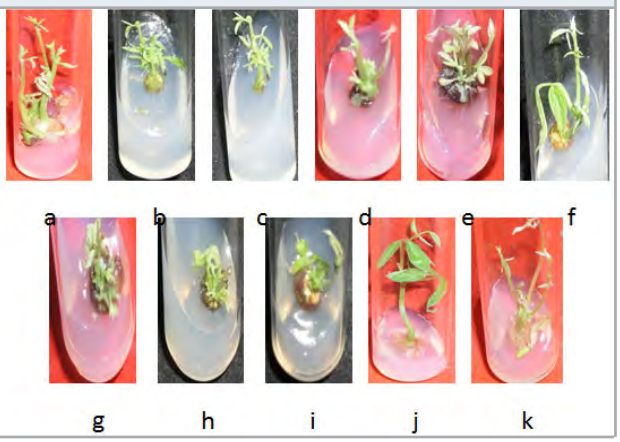

Figure 2: Multiple shoot bud induction from apical meristem explants of different cultivars of pigeonpea showing their best response in MS media supplemented with variable concentration of TDZ (in mgL^{-1}). (a)IPA-2013 (0.4), (b)IPA-3088 (0.25), (c)Pusa-9 (0.35), (d)IPA-34 (0.40), (e)IPA-204(0.30), (f)IPA-242(0.15), (g)T-7 (0.40), (h)IPA-61(0.35), (i)IPA-337 (0.25), (j)IPA-341 (0.05), (k) IPA-98-3 (0.15).

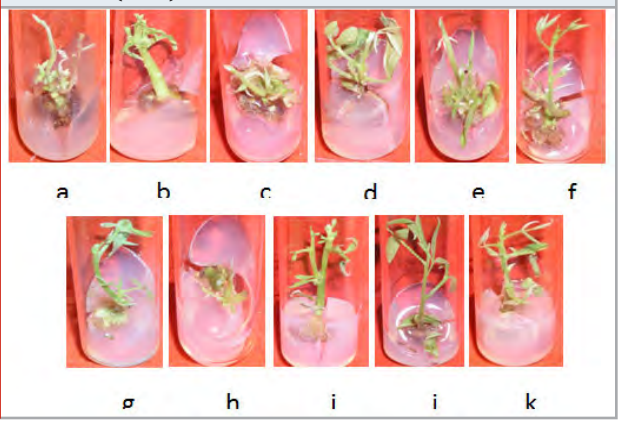

Table 2. Effect of TDZ on multiple shoot bud induction using apical meristem explants (number of shoots / explant) for eleven cultivars of pigeon pea after 4 weeks of culture with an average of 10 replicates and means with different letters differ significantly at p=0.05.

TDZ (mgL-1) → Cultivars ↓	0.05	0.1	0.15	0.20	0.25	0.30	0.35	0.40
			Number of shoots (Mean±S.D.)					
IPA-2013	2.9±0.3^{a}	1.0±0.0^{a}	3.0±0.0^{a}	3.0±0.0^{a}	3.0±0.0^{a}	2.0±0.0^{b}	2.0±0.0^{b}	4.0±0.0^{ab}
IPA-3088	3.0±0.0^{a}	1.0±0.0^{a}	3.1±0.3^{a}	3.2±0.4^{a}	6.1±0.5^{a}	4.7±0.4^{a}	4.6±0.4^{a}	4.5±0.5^{a}
Pusa-9	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	2.8±0.4^{a}	4.5±0.9^{a}	1.0±0.0^{a}
IPA-34	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	3.6±0.4^{a}
IPA-204	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	7.4±1.1^{a}	1.0±0.0^{a}	1.0±0.0^{a}
IPA-242	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
T-7	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	3.7±0.4^{a}
IPA-61	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	3.1±0.3^{a}	1.0±0.0^{a}
IPA-337	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
IPA-341	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
IPA-98-3	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0

The shoot bud induction for these cultivars with their best responsive concentration of TDZ is shown in Figure-2(a-k).

Comparative assessment of BAP, Kinetin and TDZ either singly or in combination for multiple shoot bud induction attempted for a genotype GT-102 also revealed BAP to be better hormone (Parekh et al. 2014). Multiple shoot buds obtained from apical meristem explants were subjected to rooting on full strength MS basal medium supplemented with three different hormones viz. NAA, IAA and IBA at three different concentrations namely 0.1, 0.2 and 0.3 mgL^{-1}. The response for rooting was found to be better with 0.2mgL^{-1} of NAA for most of the cultivars resulting in a maximum number of primary roots (Franklin et al. 1998). The overall response to rooting of all the eleven cultivars at three different concentrations of NAA is shown in Table-3. The response in the presence of three different concentration of IAA was also evaluated and it was found to be variable for cultivars though IPA-337 gave the best response at 0.2 mgL^{-1}of IAA. The response of rooting was poor with different concentration of

IBA for most of the cultivars in contrast to what has been reported for the cultivar Vamban-1 (Franklin et al. 1998).

The percentage acclimatization of multiple shoot buds with proper rooting in soil ranged from 25 to 75% with cultivar IPA-337, IPA-61 and IPA-204 showed 75, 70 and 65% acclimatization . The assessment of these eleven pigeonpea cultivars for direct organogenesis attempted with apical meristem explants has clearly revealed that variability in regeneration potential is genotype dependent. Further cultivar IPA-242 seems promising for direct organogenesis with apical meristem as explants source though substantial standardization for enhancing the regeneration efficiency is still needed to develop efficient regeneration protocol suitable for genetic transformation.

Table 3. Rooting responses of in- vitro regenerated shoots from apical meristem explants under different concentrations of NAA. Date recorded after 4 weeks of culture with 10 replicates for each treatment and experiment was repeated twice.

Cultivars	NAA 0.1 mg/l		NAA 0.2 mg/l		NAA 0.3 mg/l	
	% of rooting	Number of primary roots Mean±S.D.	% of rooting	Number of primary roots Mean±S.D	% of rooting	Number of primary roots Mean±S.D
IPA-2013	100	5.0±0.7	100	4.7±0.5	70	1.4±0.9
IPA-3088	80	5.7±2.9	70	2.9±1.9	80	1.6±0.8
Pusa-9	0	NR	90	4.2±1.5	80	1.9±1.0
IPA-34	100	4.6±1.4	50	1.6±1.9	0	NR
IPA-204	100	6.1±0.5	50	1.8±2.0	100	3.8±0.5
IPA-242	80	3.2±1.2	70	1.4±0.9	0	NR
T-7	100	2.0±0.0	100	6.2±0.4	0	NR
IPA-61	100	5.0±0.0	80	3.1±1.5	100	2.0±0.0
IPA-337	0	NR	80	6.4±3.2	0	NR
IPA-341	0	NR	0	NR	0	NR
IPA-98-3	0	NR	0	NR	0	NR

The percentage of rooting varied from 50 to 100% among these cultivars and IPA-337was found to be best among others for rooting with NAA (Figure-3).

Figure 3: Rooting response of apical meristem derived shoots of few cultivars of pigeonpea on MS media supplemented with different concentration of NAA (in mgL^{-1}) (a) IPA-3088 (0.1), (b) IPA-204 (0.1), (c) T-7 (0.2) and (d) IPA-337 (0.2).

CONCLUSION

Several cultivars of pigeonpea like AL 15, ICP 6917, ICP 6974, ICP 7119, ICP 7263, Vamban and GT 102 have been reported for direct organogenesis using apical meristem explants earlier. To the best of our information these selected cultivars of pigeonpea were not studied for in vitro regeneration earlier and hence an attempt has been made to decipher the potential of these cultivars for direct organogenesis exclusively for apical meristem as explants. Among the three growth hormones BAP, TDZ and kinetin studied for in vitro regeneration among these cultivars, multiple shoot bud induction and regeneration was found to be better with higher concentration of BAP as reported earlier. Genotype-dependent response for organogenesis under the influence of variable concentration of growth regulators was observed for these cultivars. The best responsive cultivars for multiple shoot bud induction and in vitro regeneration under variable concentration of BAP, Kinetin and TDZ treatments were IPA-242, IPA-2013 and IPA-204 respectively. A maximum of 7 buds observed with IPA-242 at higher concentration of BAP has immense potential for developing efficient regeneration protocol using apical meristem explants which could be further tested for its amenability for genetic transformation.

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Diversity of Butterflies in Tipeshwar Wildlife Sanctuary of Maharashtra, India

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ABSTRACT

Butterflies are considered as the best indicators of the health of any specified terrestrial ecosystem. They are key components in maintaining ecological dynamics of the protected areas and protected areas provides major support systems for maintaining their diversities. This scientific exercise is undertaken from 1 Dec. 2017 to 30 Nov. 2018 to explore butterfly diversity in Tipeshwar Wildlife Sanctuary, a protected area spread over 148.63 km^2 and located at 78°20'22'' to 78°47'56'' East and 19°50'59'' to 19°55'44'' North situated in the Deccan peninsular of Central Indian landscape. Varieties of plant species of this dry deciduous forest and seasonal variation in floral composition of this wildlife sanctuary attract verities of butterfly species.97 species of butterflies belong to 64 genera of 5 families dominated by family Nymphalidae (34.02%), Lycaenidae (27.83%) followed by Pieridae (19.59%), Hesperiidae (11.34%) and Papilionidae (7.21%) are recorded.It appears that the butterfly abundance increased from monsoon to winter while decreased in summer and pre-monsoon possibly due to the unavailability of nectar and the changes in temperature and humidity of this protected area. Butterflies are considered as an important model group in understanding ecology of a particular landscape. This research exercise will help in understanding ecology of this protected area and prove to be the important biological tool in devising the strategies for sustainable conservation of wildlife of this protected area and similar geographical regions

KEY WORDS: BUTTERFLY, BIOINDICATORS, DIVERSITY, POPULATION DYNAMICS, TIPESHWAR.

INTRODUCTION

The butterflies are the most attractive elements of the biological diversity of the universe (Losey and Vaughan 2006). They are beautifully coloured, ecologically important insects belong to order Lepidoptera of class insecta. There are 1.5 million identified animal species harbour on the earth, class insecta alone contributes near about 0.8 million species whereas butterfly and moth shares 0.14 million species.More than 1700 species of butterflies are recorded from across the globe, of this India alone contributes 1504 (Gaonkar 1996; Smetacek 1992; Kunte 2009; Roy et al., 2010). Central India is home of 1400 species of butterfly (A Biodiversity Atlas- India Website), 167 amongst them are reported from Vidarbha, (Triple 2011). 111 species of butterfly are reported in and around Tadoba National Park of central India (Tiple 2010). Diversity of butterflies in Karhandla region of Umred-Karhandla wildlife sanctuary, studied by Gajbe (2016) and 53 species of butterflies belong to 34 genera of 5 families are recorded inhabiting in this protected area. Butterflies show co-evolutionary relationship with the plants and perform prominent roles in pollination (Tiple et al 2006; Tiple 2018).

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As pollinators butterflies are valuable creatures in maintaining the population dynamics of floral composition of natural and man-made ecosystems. Klein et al., (2008) have estimated that 35% of food use by human contributed from crop pollinated by insects (majorly by butterflies). As an integral part of prey-predator system they play major role in maintaining ecological balance in any type of ecosystem. As a bio-indicators, butterflies are useful in monitoring the ecological imbalance due to pollution, uncontrolled exploitation of natural resources, illegal encroachment and significant in studying the impact of rapid urbanisation on ecology in developing countries like India, (Khairunnisa et al., 2015). Global climate change has detrimental effect on butterfly diversity and its distribution as they are very specific in ecological requirements such as temperature, humidity, food plants and egg-laying habitats, (Forister and Shapiro 2003; Gonzalez-Megias et al., 2008).

Temperature and relative humidity are the important factors in distribution and assemblage of Butterfly species (Gupta et al., 2019). Butterflies are considered as the best indicators of the health of any specified terrestrial ecosystem (Thomas 2005; Bonebrake et al., 2010) and therefore treated as an important model group in understanding ecology of any landscape and to draw strategies for conservation accordingly (Watt and Boggs 2003; Ehrlich and Hanski 2004; Mukherjee et al., 2015). They are key components in maintaining ecological dynamics of the protected areas and protected areas are major support systems for maintaining their diversities. Distribution and variation in butterfly diversity changes in heterogeneous habitats with different ecological parameters (Suryanarayana et al., 2018).

This research exercise was aimed to estimate butterfly diversity in the Tipeshwar Wildlife Sanctuary, Maharashtra, India. Varieties of plant species of this dry deciduous forest and seasonal variation in floral composition of this wildlife sanctuary attract verities of species of butterfly. The results of this research exercise will help in understanding ecology of Tipeshwar Wildlife Sanctuary and it will prove to be the important biological tool in devising the strategies conservation of wildlife of this protected area and similar geographical regions by understanding ecological role of these flying beauties.

MATERIAL AND METHODS

Study area: The Tipeshwar Wildlife Sanctuary situated in Yavatmal District of Indian state of Maharashtra. It is located between of 78°20'22'' to 78°47'56'' East and 19°50'59'' to 19°55'44'' North with total area of 148.63 sq. km. It constitutes compact patches of dense forest cover with meadows and a seasonal wetland. It has great utility from the point of view of wildlife and bio-diversity conservation. The main portion of this protected area constitutes the dry teak bearing forest. The climatic condition of this area is characterized by a hot summer, well-distributed rainfall during the south-west monsoon season and generally dry weather during rest of the year. The cold season is from December to February (Yavatmal Gazetteer 2019).

Survey method: The butterflies were observed from the study sites for a period of 1 year between 1 Dec. 2017 to 30 Nov. 2018. During the survey, an efficient protocol was adopted. The survey was made using a "Pollard Walk" method (Pollard 1977; Pollard and Yates 1993) with necessary modifications. Study area was visited twice a month from early morning (8:00 AM) to afternoon (11:00 AM) during good weather periods.

Species identification: After detection, a butterfly was photographed in field (Nikon D7100+ Nikkor 105 micro lense; Nikon Inc., Tokyo, Japan) and identified with the help of visible structural features. For identification and comparative studies of observed specimens, keys and methods suggested by Evans (1932), Wynter-Blyth (1957), Haribal (1992), Kunte (2000) and Kehimkar (2008) were adopted.

Data analysis: Species occurrence analysis was carried out by Microsoft excel program with using the following formulas. Relative Dominance (RD) of species was calculated as [RD=Ni × 100/Nt] where, Ni is number of individuals of species and Nt is total number of individuals all species (Basavarajappa 2006; Joshi 2014). Relative Occurrence (RO) of family was calculated as [RO= Ns × 100/Nt] where, Ns is number of species of each family and Nt is total number of all species (Basavarajappa 2006; Joshi 2014). Mean percent occurrence (M%) for month was calculated as [M% = Nm × 100 /Nt] where, Nm is number of individuals in each month and Nt is total number of individuals during complete study tenure (Basavarajappa 2006; Joshi and Tantarpale 2016). The mean values of the pooled species occurrence data were used to calculate the monthly diversity of and to categorize the local status of species.

The diversity assessment enabled highlighting the observed species richness pattern of the saurian species. The diversity indices were quantified with the help of PAST Version 1.60 software (Palaeontological As so., Norway; Hammer et al., 2001). The species diversity was calculated using Shannon diversity index that calculated as $[H' = -\sum_{i=1}^{R} Pi \log Pi$ where Pi is proportion of the first species which is given by Pi= ni/N (Magurran 1988); species richness was obtained by using Margalef equation [R= (S-1)/ log N], Where, R is Index of species richness, S is Total number of species and N is Total No. of individuals (Magurran 1988); while Species equitability was determined by equation of Pielou [J= N_1/N_0] where N_1 is Number of abundant species in the sample and N0 is Number of species in the sample (Hammer et al., 2001). The similarity association matrix upon which the cluster based was computed using the nearest neighbour pair linkage algorithm of Euclidean distance index for presence and absence data (Hammer et al., 2001). The differences between the diversity and evenness indices of with species occurrence among different study months were statistically analyzed by using Analysis of variance (ANOVA). The statistical analyses were performed

following Zar (1999) using the SPSS version 10 (SPSS Inc., Chicago, Il, USA; Kinnear and Gray 2000).

RESULTS AND DISCUSSION

During this study, 97 butterfly species under five families were observed in study area (Table 1). Based on value of butterfly relative dominance in study area, 19.59 % species was categorized as abundant species whereas 44.32 % species was common, 12.37 % species was frequent, 15.46 % was occasional, and 8.24 % species was rare (Figure 1). The maximum number of butterfly species were recorded under family Nymphalidae (34.20 %), Lycaenidae (27.83 %) followed by Pieridae (19.58 %), Hesperiidae (11.34 %) and Papilionidae (7.21 %) (Figure 2).

Table 1. Butterfly diversity in the Tipeshwar Wildlife Sanctuary, Maharashtra, India during 1 Dec. 2017 to 30 Nov. 2018

Common Name	Scientific Name	Pictures	Local Status	IUCN status	Relative Dominance	
1 Family: Papilionidae						
Tailed Jay	*Graphium agamemnon* (Linnaeus, 1758)		Common	NE	1.115	
Common Jay	*Graphium doson* (Felder and Felder, 1864)		Common	NE	1.145	
Common rose	*Pachliopta aristolochiae* (Fabricius, 1775)		Common	LC	1.038	
Crimson rose	*Pachliopta hector* (Linnaeus, 1758)		Common	NE	0.980	SCH.I Part IV
Lime Butterfly	*Papilio demoleus* (Linnaeus, 1758)		Abundant	NE	1.379	
Common Mormon	*Papilio polytes* (Linnaeus, 1758)		Abundant	NE	1.291	
Spot Swordtail	*Graphium nomius* (Esper, 1793)		Occasional	NE	0.624	
Family: Pieridae						
Common Albatross	*Appias albino* (Fabricius, 1775)		Common	NE	1.084	SCH.II Part II
Indian Pioneer	*Belenois aurota* (Fabricius, 1793)		Abundant	NE	1.467	
Common Emigrant	*Catopsilia pomona* (Fabricius, 1775)		Common	NE	0.959	
Mottled Emigrant	*Catopsilia pyranthe* (Linnaeus, 1758)		Common	NE	0.917	

Table 1 Continue

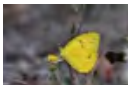
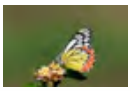
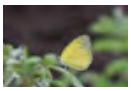
Common Gull (Fabricius, 1775)	*Cepora nerissa*		Common	NE	0.931	SCH.II Part II
Small salmon Arab	*Colotis amata* (Butler, 1870)		Occasional	NE	0.531	
Large Salmon Arab	*Colotis fausta* (Olivier, 1804)		Rare	NE	0.237	
Crimson Tip	*Colotis danae* (Fabricius, 1775)		Occasional	NE	0.537	
Small Orange Tip	*Colotis etrida* (Boisduval, 1836)		Common	NE	1.096	
White Orange Tip	*Ixias Marianne* (Cramer, 1775)		Common	NE	1.072	
Yellow Orange Tip	*Ixias pyrene* (Linnaeus, 1764)		Occasional	NE	0.713	
Common Jezebel	*Delias eucharis* (Drury, 1773)		Common	NE	1.083	
One Spot Grass Yellow	*Eurema andersoni* (Moore, 1865)		Abundant	LC	1.298	
Three Spot Grass Yellow	Eurema blanda (Boisduval, 1836)	Observed in field	Frequent	NE	0.823	
Small Grass Yellow	*Eurema brigitta* (Stoll, 1780)		Common	LC	1.181	
Common Grass Yellow	*Eurema hecabe* (Linnaeus, 1758)		Abundant	NE	1.349	
Spotless Grass Yellow	*Eurema laeta* (Boisduval, 1836)		Abundant	NE	1.419	
Psyche	*Leptosia nina* (Fabricius, 1793)		Occasional	NE	0.734	
Common Wanderer	*Pareronia valeria* (Cramer, 1776)		Common	NE	1.163	
Family: Nymphalidae						
Tawny Castor	*Acraea violae* (Fabricius, 1775)		Common	NE	1.198	
Angled Castor	*Ariadne ariadne* (Linnaeus, 1763)		Abundant	NE	1.020	
Common Castor	*Ariadne merione* (Cramer, 1779)		Common	NE	1.123	

Table 1 Continue

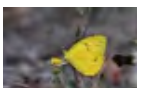
Plain Tiger	*Danaus chrysippus* (Linnaeus,1758)		Abundant	NE	1.449	
Striped Tiger	*Danaus genutia* (Cramer, 1779)		Abundant	NE	1.324	
Common Crow	*Euploea core* (Cramer, 1780)		Common	LC	1.134	
Double Branded crow	*Euploea Sylvester* (Fabricius, 1793)		Occasional	NE	0.549	
Baronet	*Euthalia nais* (Cramer, 1779)		Common	NE	0.974	
Common Baron	*Euthalia aconthea* (Cramer, 1777)		Rare	NE	0.204	
Great Eggfly	*Hypolimnas bolina* (Linnaeus, 1758)		Common	NE	1.111	
Danaid Eggfly	*Hypolimnas misippus*		Occasional	NE	0.713	
Common Jezebel	*Delias eucharis* (Drury, 1773) (Linnaeus, 1764)		Common	NE	0.999	SCH.II Part II
Peacock Pansy Grass Yellow	*Junonia almana* (Linnaeus, 1758)		Abundant	LC	1.167	
Grey Pansy	*Junonia atlites* (Linnaeus, 1763)		Common	NE	1.202	
Yellow Pansy	*Junonia hierta*		Common	LC	1.157	
Lemon Pansy	*Junonia lemonias* (Linnaeus, 1758)		Abundant	NE	1.364	
Spotless Grass Yellow	*Eurema laeta* (Boisduval, 1836)		Abundant	NE	1.419	
Psyche	*Leptosia nina* (Fabricius, 1793)		Occasional	NE	0.734	
Common Wanderer	*Pareronia valeria* (Cramer, 1776)		Common	NE	1.163	
Family: Nymphalidae						
Tawny Castor	*Acraea violae* (Fabricius, 1775)		Common	NE	1. 198	
Angled Castor	*Ariadne ariadne* (Linnaeus, 1763)		Common	NE	1.020	

Table 1 Continue

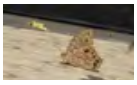
Common Castor	*Ariadne merione* (Cramer, 1779)		Common	NE	1.123	
Plain Tiger	*Danaus chrysippus* (Linnaeus,1758)		Abundant	NE	1.449	
Striped Tiger	*Danaus genutia* (Cramer, 1779)		Abundant	NE	1.324	
Common Crow	*Euploea core* (Cramer, 1780)		Common	LC	1.134	
Double Branded crow	*Euploea Sylvester* (Fabricius, 1793)		Occasional	NE	0.549	
Baronet	*Euthalia nais* (Cramer, 1779)		Common	NE	0.974	
Common Baron	*Euthalia aconthea* (Cramer, 1777)		Rare	NE	0.204	
Great Eggfly	*Hypolimnas bolina* (Linnaeus, 1758)		Common	NE	1.111	
Danaid Eggfly (Linnaeus, 1764)	*Hypolimnas misippus*		Common	NE 0.999	Part II	SCH.II
Peacock Pansy	*Junonia almana* (Linnaeus, 1758)		Common	LC	1.167	
Grey Pansy	*Junonia atlites* (Linnaeus, 1763)		Common	NE	1.202	
Yellow Pansy	*Junonia hierta* (Fabricius, 1775)		Common	LC	1.157	
Chocolate Pansy	*Junonia iphita* (Cramer, 1779)		Common	NE	1.011	
Lemon Pansy	*Junonia lemonias* (Linnaeus, 1758)		Abundant	NE	1.364	
Blue Pansy	*Junonia orithya* (Linnaeus, 1764)		Abundant	NE	1.480	
Common Evening Brown	*Melanitis leda* (Linnaeus, 1758)		Abundant	NE	1.303	
Dark Evening Brown	*Melanitis phedima* (Cramer, 1780)		Occasional	NE	0.625	
Common Bush Brown	*Mycalesis perseus* (Fabricius, 1775)		Frequent	NE	0.789	

Table 1 Continue

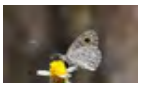
Long Brand Bush Brown	*Mycalesis visala* (Moore, 1858)		Occasional	NE	0.703	
Common Sailor	*Neptis hylas* (Linnaeus, 1764)		Common	NE	0.968	
Common Leopard	*Phalanta phalantha* (Drury, 1773)		Common	LC	1.051	
Blue Tiger	*Tirumala limniace* (Cramer, 1775)		Common	NE	1.135	
Commander	*Moduza procris* (Cramer, 1777)		Common	NE	1.190	
Painted Lady	*Synthia cardui* (Linnaeus, 1764)		Common	NE	0.950	
Joker	*Byblia ilithyia* (Drury, 1773)		Common	NE	0.941	
Common Three Ring	*Ypthima asterope* (Klug, 1832)		Common	NE	1.193	
Large Three Ring	*Ypthima nareda* (Kirby, 1871)		Frequent	LC	0.868	
Anomalous Nawab	*Polyura agrarian* (Linnaeus, 1764)		Occasional	NE	0.703	
Common Nawab	*Polyura athamas* (Drury, 1773)		Occasional	NE	0.502	SCH.II Part II
Black Rajah	*Charaxes solon* (Fabricius, 1793)		Rare	NE	0.205	SCH.II Part II
Towny Rajah	*Charaxes bernardus* (Fabricius, 1793)		Rare	NE	0.276	SCH.II Part II
Family: Lycaenidae						
Pointed Ciliate Blue	*Anthene lycaenina* (C. Felder, 1868)		Occasional	NE	0.699	SCH.II Part II
Large Oak Blue	*Arphopala amantes* (Hewitson, 1862)		Rare	NE	0.197	
Dull Babool Blue	*Azanus uranus* (Butler, 1886)		Frequent	NE	0.831	
Bright Babool Blue	*Azanus ubaldus* (Stoll, 1782)		Common	NE	0.953	
Lime Blue	*Chilades lajus* (Stoll, 1780)		Common	NE	1.192	

Table 1 Continue

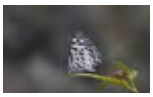
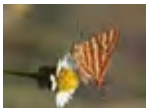
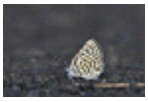
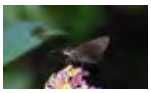
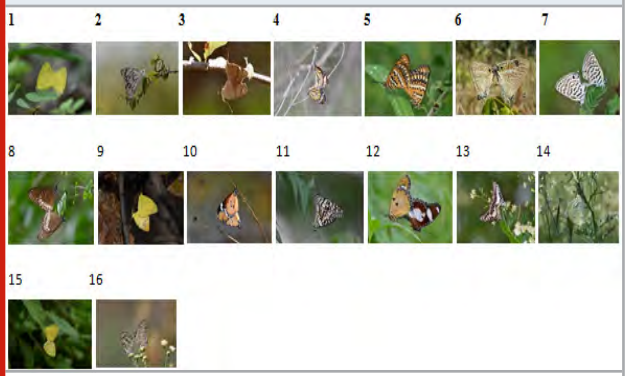
Gram Blue	*Euchrysops cnejus* (Fabricius, 1798)		Common	NE	1.051	SCH.II Part II
Pea Blue	*Lampides boeticus* (Linnaeus, 1767)		Common	NE	1.136	SCH.II Part II
Zebra Blue	*Leptotes plinius* (Fabricius, 1793)		Abundant	NE	1.234	
Tailless Line Blue	*Prosotas dubiosa* (Semper, 1879)		Common	NE	1.062	SCH.II Part II
Common Line Blue	*Prosotas nora* (Felder, 1860)		Common	NE	1.172	
Guava Blue	*Virachola isocrates* (fabricius, 1793)		Occasional	NE	0.688	SCH.I Part IV
Dark Grass Blue	*Zizeeria karsandra* (Moore, 1865)		Abundant	NE	1.312	
Lesser Grass Blue	*Zizina otis* (Fabricius, 1787)		Abundant	NE	1.233	
Tiny Grass Blue	*Zizula hylax* (Fabricius, 1775)		Common	NE	1.049	
Plum Judy	*Abisara echerius* (Moore, 1901)		Occasional	NE	0.718	
Common Pierrot	*Castalius rosimon* (Fabricius, 1775)		Frequent	NE	0.899	SCH.I Part IV
Forget-Me-Not	*Catochrysops strabo* (Fabricius, 1793)		Abundant	NE	1.336	
Plains Cupid	*Luthrodes pandava* (Horsfield, 1829)		Frequent	NE	0.854	
Indian cupid	*Cupido lacturnus* (Godart, 1824)		Frequent	NE	0.811	
Grass Jewel	*Freyeria trochylus* (Freyer, 1845)		Common	NE	1.153	
Common Cerulean	*Jamides celeno* (Cramer, 1775)		Frequent	NE	0.824	
Indian Red Flash	*Rapala airbus* (Fabricius, 1787)		Rare	NE	0.233	
Slate Flash	*Rapala manea* (Hewitson, 1863)		Rare	NE	0.137	SCH.I

Table 1 Continue

Common Silverline	*Spindasis vulcanus* (Fabricius, 1775)		Frequent	NE	0.829	
Common Shot Silverline	Spindasis ictis (Hewitson, 1865)		Occasional	NE	0.591	
Rounded Pierrot	*Tarucus extricates* (Kollar, 1848)		Abundant	NE	1.217	
Peacock Royal	*Tajuria cippus* (Fabricius, 1775)		Rare	NE	0.170	SCH.II Part II
Family: Hespiridae						
Brown awl	*Badamia exclamationis* (Fabricius, 1775)		Abundant	LC	1.397	
Common Banded Awl	*Hasora chromus* (Cramer, 1780)		Frequent	NE	0.828	
Rice swift	*Borbo cinnara* (Wallace, 1866)		Abundant	NE	1.489	
Small branded swift	*Pelopidas mathias* (Fabricius,1798)		Abundant	NE	1.279	
Conjoined Swift	*Pelopidas conjuncta* (Moore, 1878)		Common	NE	0.916	
Paintbrush Swift	*Baoris farri* (Moore, 1878)		Frequent	NE	0.846	SCH. IV
Common Straight Swift	*Parnara guttatus* (Bremer and Gray, 1853)		Common	LC	1.151	
Indian Palm bob	*Suastus gremius* (Fabricius, 1798)		Common	NE	0.935	
Dark Palm-Dart	Telicota ancilla (Moore, 1878)		Common	NE	1.090	
Indian skipper	Spialia galba (Fabricius, 1793)		Frequent	LC	0.782	
Grass Demon	Udaspes folus (Cramer, 1775)		Occasional	NE	0.661	

Breeding Records of some butterflies from 1 Dec. 2017 to 30 Nov. 2018 in Tipeshwar Wildlife Sanctuary



1.Small Grass Yellow 2.Pioneer 3.Blue Pansy 4.Striped Tiger 5.Joker 6.Pea Blue 7.Rounded Pierrot 8.Common Crow 9.Yellow Orange tip 10.Plain Tiger 11.Lime Butterfly 12.Danied Egg fly 13.Common Jay 14.Common Emigrant 15.Common Grass Yellow 16.Zebra Blue

Figure 1: Relative occurrence of butterfly Species from 1 Dec. 2017 to 30 Nov. 2018 in Tipeshwar Wildlife Sanctuary

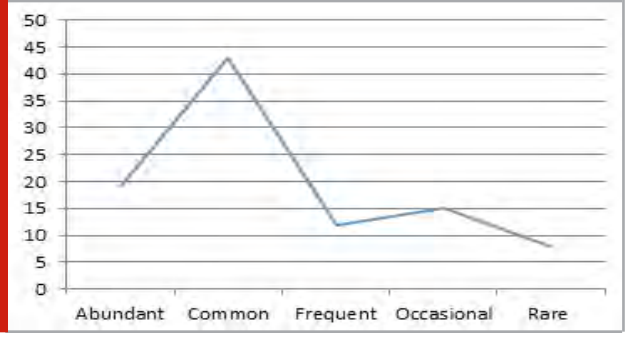
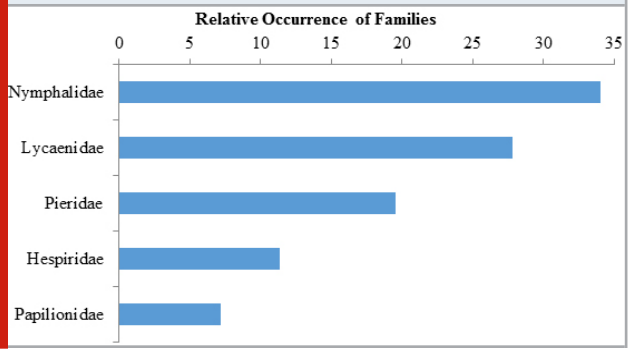


Figure 2. Relative dominance of butterfly families studied from 1 Dec. 2017 to 30 Nov. 2018 in the Tipeshwar Wildlife Sanctuary, Maharashtra, India



A dendrogram developed by Euclidean distance cluster analysis was observed to be multifaceted and showed variation in the level of similarity in the number of butterfly species in 12 months. The months with the minimum to moderate number of species belong to one cluster, whereas the rest of the months with moderate to maximum number of species formed another cluster (Figure 3). It appears that the butterfly abundance increased from monsoon to winter while decreased in the summer and pre-monsoon possibly due to the unavailability of nectar and the change in temperature and humidity of the habitats concerned.

Figure 3: Dendrogram showing similarity in number of butterfly species composition among the studied month during 1 Dec. 2017 to 30 Nov. 2018

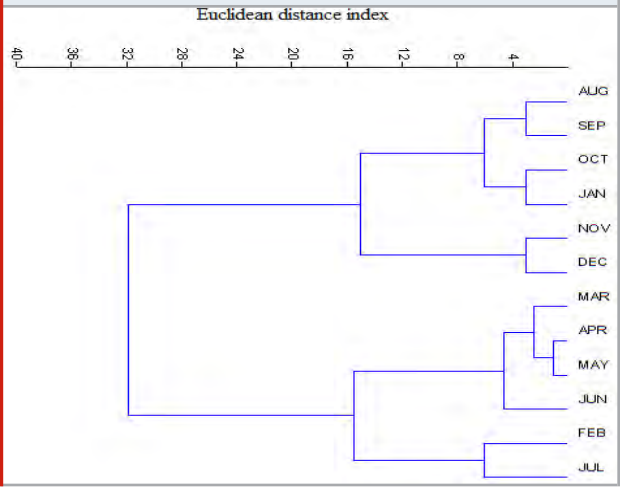
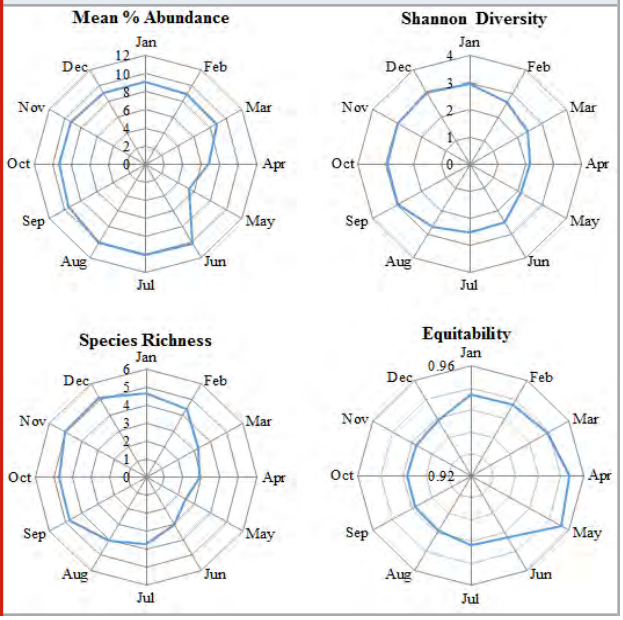


Figure 4: The values of the diversity indices in different months from 1 Dec. 2017 to 30 Nov. 2018 observed through the random sampling of butterflies in the Tipeshwar Wildlife Sanctuary, Maharashtra, India



Observations on the monthly variations of butterfly abundances indicate patterns of peak from June to November while a low from December May and from (Figure 3). Mean percent abundance of butterflies was significantly different ($F = 121.8$, $df = 11$, $p < 0.05$); Shannon diversity values of butterflies were significantly different ($F = 148.2$, $df = 11$, $p < 0.05$); species evenness among different months was significantly different

(F= 142.1, df = 11, $p < 0.05$) while species richness among the study months was significantly different (F = 156.4 df = 11, $p < 0.05$). A trend in mean % abundance, Shannon diversity, species richness and species equitability showed the contradictory patterns (Figure 4).

The butterflies are the ecologically important organisms that serves as indicators of environmental conditions (Stefanescu et al., 2004). Observations on the butterfly diversity provide the information about variations in the species richness and the abundance in relation to the vegetation and associated landscapes (Öckinger and Smith 2006; Öckinger et al., 2006; Mutmainnah and Santosa 2019). In this context, the diversity of butterflies in the Tipeshwar Wildlife Sanctuary, Maharashtra, India was studied during Dec. 2017 to 30 Nov. 2018. Varieties of plant species of this dry deciduous forest and seasonal variation in floral composition of this protected area attract varieties of species of butterfly. The earlier studies showed that heterogeneity of the habitats in terms of the available plant species supports the rich butterfly diversity (Kuussaari et al., 2007; Mukherjee et al., 2015). Butterfly diversity even in the agricultural landscape contrast to the urban and suburban regions show that the richness increased with the availability of the green space and the heterogeneity of the habitats in terms of the available plant species (Öckinger et al., 2009; Mukherjee et al., 2015).

Consistent with these studies the present observation records a total of 97 species belonging to five families.It was observed that the family Nymphalidae represented by 18 genera and 33 species was the most dominant followed by Lycaenidae (23 genera, 27 species), Pieridae (10 genera, 19 species), Hesperiidae (10 genera, 11 species), and Papilionidae (3 genera, 7 species). The maximum number of butterfly species was recorded under family Nymphalidae and Lycaenidae followed by Pieridae, Hesperiidae and Papilionidae. Relative dominance of butterfly species studied is, 19.58 % species was categorized as abundant whereas 44.32 % species as common, 12.37 % species as frequent, 15.46 % as occasional, and 8.24 % species was rare.

Out of these 97 butterfly species studied, 15 species specified under Indian Wildlife (Protection) Act, 1972 were encountered in good numbers. The butterflies *Pachliopta hector Castalius rosimon* and *Virachola isocrates* are placed in Schedule I Part IV, the species *Appias albino, Cepora nerissa, Hypolimnas misippus, Polyura athamas, Charaxes bernardus, Anthene lycaenina, Charaxes solon, Euchrysops cnejus, Lampides boeticus*. *Prosotas dubiosa* and *Tajuria cippus* are protected under Schedule II Part II, while *Baoris farri* is categorized as Schedule IV. It is observed that the species diversity and its abundance is high from monsoon to early winter and decline from early summer onwards due to the reduction in moisture and scarcity of host plant species. Temperature and relative humidity are the important factors in distribution and assemblage of Butterfly species (Gupta et al., 2019).

Observations on the monthly variations of butterfly encounters indicates that population is high in monsoon months and declining towards summer while diversity is at peak from August to December while a low from January to May. The present observations remain consistent with the records and views of the butterfly species in different parts of the world (Wilson et al., 2004; Tiple et al., 2006; Sodhi et al., 2010; Tiple 2018). The butterfly species observed in the present study remained similar to the available observations on the species in different parts of India bearing similar landscape patterns (Roy et al., 2012; Harsh 2014; Saikia 2014; Mukherjee et al., 2015). Dominance of the butterflies of the family Nymphalidae as revealed through the present study is similar to that observed in other parts of the country (Mutmainnah and Santosa 2019).

In parity with the species diversity observed in Tipeshwar Wildlife Sanctuary, Maharashtra, India, it may be assumed that the butterflies play diverse functional roles for the sustenance of the ecosystems. The richness in species composition in study area was also prominent in present investigation. The availability of the vegetation, seasonal wetland and allied factors render stability to the butterfly population and assemblages in the landscapes are possibly important contributors to the observed variations in the butterfly species. The present diversity study is confined to a limited area and selected habitats. There is, in the future, a chance of more species being reported because of few pockets and habitats in the studied area requiring more extensive exploration.

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Soaking Kinetics, Dimensional Analysis and Germination Indices of Kidney Bean Under Different Chemical Stresses

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ABSTRACT

The aim of this study was to observe the effect of chemical stress induced by different salts on the soaking kinetics and germination indices of kidney bean. Water uptake of seed grains greatly affected by the ionic composition of soaking solution used. Water absorption rate and capacities were studied in relation with Peleg's model using hydration data and plotting graphs at different time interval of soaking. Dimensional analysis like spherecity (Ø), aspect ratio (Ra), volume (V), and surface area (S) was observed using mathematical equations based on linear measurements like length, width and thickness. Effect of salt stress on the germination was observed using various parameters like germination percentage (G), vigor value, mean germination time (t^-), mean germination rate (MR), and coefficient of variation of germination (CV). Grains soaked in different chemicals resulted in the different absorption pattern and Peleg's constants. Peleg's rate constant was lower in NaOH (1.46×10^{-3}) and capacity constant was lower in distilled water (2.94×10^{-2}). Dimensional characteristic exhibited their dependence on water uptake properties and varied to some extent due to difference in absorption capacities. Germination data also varied for seed grains soaked under different chemical stress. Germination percentage and vigor value were greatly affected due to chemical stresses. Germination percentage was higher in distilled water (78%), whereas vigor value was higher in $MgCl_2$ (0.5%). It was concluded that, salt stress under different chemicals greatly affected the water absorption pattern and as result variation in the germination pattern among different groups was observed.

KEY WORDS: SALINITY, SALT STRESS, SOAKING KINETICS, PELEG'S CONSTANTS, GERMINATION INDICES.

INTRODUCTION

Salt stress is the foremost abiotic stress in the agriculture. It induces adverse effects on the initial developmental stages in plant life cycle. These abiotic stresses were found problematic in seed germination, establishment, reproductive and vegetative growth (Zhu, 2016). Salt stresses may inhibit seed germination and post germinated growth. Germination is correctly defined by Bewley and Black (1994) as the process from imbibition to embryo protrusion in seed grains and post- germination involves the processes after embryo protrusion which leads to plant formation. Thus, soaking kinetics under different chemical stresses needed to be studied and is of practical importance for breeders in the development of salt resistant seeds. Mathematical models based on the theories and diffusion models (Corzo et al. 2004) to interpret absorption data in the form of moisture vs. time was studied periodically for better understanding of absorption behavior of seed grains. According to Rastogi et al. (2000), various researchers used different kind of mathematical models and expressions to understand the rehydration or absorption kinetics of different material (Sanju et al. 1999). Peleg (1988), observed a bivariate

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mathematical model to observe its potential implication in absorption data (Peleg 1988).

Mixture of salts present in the soil has cumulative effect on the growth of seedling. Cramer (1985) reported that salts affect the functioning and cell wall of seeds. Studies have shown that the influx and efflux of cytosolic solutes were greatly affected by the permeability of membranes which in turn affected by the presence of ions present around (Allen et al. 1995). Apart from permeability salt components could affect the hardening of cell wall (Neumann et al. 1994) and water conductance of plasma membrane (Azaizeh et al. 1992). These changes in the cell membrane and cell wall affect the moisture absorption and germination of seed by affecting the water potential of inner cellular material and cell extensibility (Tobe et al. 2004). Research has also shown the utilization of germinated grains in composite flours to enhance nutrition index of bakery products (Sibian et al. 2020).

Calculating the germination percentage and vigor is not enough to portray the germination profile of seed. Different germination parameters were required to plot the germination characteristic of seed. Germination and vigor test provide only the insight in the performance profile of seed lot and effects the seed storage conditions. Therefore, germination index has been proposed by Riss and Bang-Olsen (1991) to characterize the germination rate. Due to the differences in the germination times of seed among group, the concept of mean germination time has been introduced by Edward (1932) as the concept of extreme times for germination i.e. time for first and last seed to germinate (Tobe et al. 2004).

Prodanov et al. (2004) and Ali et al. (2009) observed the effect of alkalinity and salinity on the composition of certain grains but not much work has been done on the effect of different soaking solutions on the absorption kinetics, and the engineering aspect during soaking along with detailed post germination evaluation of the legume seed. In this research, efforts have been made to observe the effect of individual salts of Na+ and Mg2+ on the soaking kinetics and germination of kidney bean (*Phaseolus vulgaris*-Cranberry Group). To observe the effect in seed-water relationship, the computational data of different salt stresses and their interaction with the kidney bean was established. Three groups of salt stress treatments were used in comparison with distilled water. Research was distributed into 3 sections viz. soaking and absorption kinetics, dimensional analysis after soaking and computational analysis of germination (Ali et al. 2009).

MATERIAL AND METHODS

Preparation of raw material and soaking solutions: Viable grains of kidney bean (*Phaseolus vulgaris-Cranberry* Group) were procured from local farmer of Amritsar, Punjab. The age of grains were not more than 6-12 months and were stored in appropriate environment. Different soaking solution groups were prepared viz. NaOH-0.1%; $NaHCO_3$-0.5%; $MgCl_2$-0.5% and distilled water. The concentration of solutions was based on the trial work conducted to optimize the minimal effect of solutions on seed germination.

Soaking of seeds and collection of hydration data: Four sets of pre-weighed seed grains samples (10 grams each) (n=3) were taken and soaked in 250 ml solutions (NaOH-0.1%; $NaHCO_3$-0.5%; $MgCl_2$-0.5%; distilled water) each at 40±2°C in water bath. Temperature of soaking solution was kept constant at 40°C, which was observed as optimum soaking temperature to facilitate the water uptake without exhibiting the boiling effect (Turhan et al. 2002). Measurement of gain in moisture content was observed at different intervals until saturation point was achieved. The hydrated seeds were blotted free of excess surface moisture with tissue paper and then weight was determined using analytical weighing balance (Shimadzu-AUW-D Series). The gain in weight (initial moisture (db) + water uptake) gave the amount of water uptake at that specific time and graphs were plotted between time and moisture gain. The rate of moisture absorption at specific time was calculated as dM/dt i.e. rate of change of moisture at specific time "t".

Peleg's constants (K_1 and K_2): Values of moisture content at different time period were reported as discussed above and then analyzed for the variables of Peleg's equation in computer based statistical software (Statsoft Statistic ver. 10.0). Moisture content on dry basis was used for the calculation of Peleg's constants K_1 and K_2 as per mathematical expression of Peleg (1988):

$$M_t = Mo \pm \frac{t}{K_1 + K_2 . t}$$

Where, M_t= moisture content at known time (t) (% db), M_o= initial moisture content (% db), t= soaking time, K_1= Peleg's rate constant, K_2= Peleg's capacity constant.

Dimensional analysis after soaking of seeds: Seeds from each soaking group were randomly measured for Length (L), width (W), and thickness (T). Raw (un-soaked) grains were also measured for dimensional analysis and were compared to observe the effect of soaking. Measurements included the morphological features of grains, which were measured by using digital Vernier caliper (Aerospace 150 mm digimatic) with 0.01 mm accuracy. The average of each measurement group was taken for further dimensional parameters like spherecity, surface area, aspect ratio, volume of seed. Spherecity (Ø) is the criteria used to describe the shape of seed. The spherecity was calculated using following equation:

$$Spherecity\ (Ø) = \frac{(L + W + T)^{1/3}}{L}$$

Similar to spherecity, aspect ratio is also considered as important criteria to characterize shape of seed grains. The aspect ratio (Ra) of seed grains was calculated

as described by Mohsenin (1980) using following mathematical expression:

$$R_a = \frac{W}{L}$$

Surface area of seed grain was calculated using following equations as described by Jain and Bal (1997).

$$S = \frac{BL^2}{(2L - B)}$$

Where,

$L = \text{Length}$,

$$B = \sqrt{\text{Width.Thickness}}$$

The measurement of volume and surface area of seed grains is an important criterion to estimate the permeability and moisture absorption pattern of seed. Surface area provide the area of contact of water molecules with the surface of seed grains. Length, width and thickness of seed grains were used as function for the measurement of volume of seed grains. The volume of seed (V) was calculated using following equations as described by Jain and Bal (1997):

$$V = 0.25\left\{\left(\frac{\pi}{6}\right)L(W+T)^2\right\}$$

Germination of seed grains: Kidney bean seeds were cleaned and soaked separately in respective solutions. Germination of grains was carried out as per the method described by Sibian et al. (2016) in double chambered seed germinator (Alpha chem 956) under controlled conditions (Temp: 28±1°C /RH: 45±5%) for 96h. Data was generated by repeatedly counting of seeds on different time intervals and emergence of plumule was taken as indicator of germination.

Analysis of seed germination: Germination percentage (G) is the measure of proportion of number of seeds germinated during assay. Vigor value is an important agronomic feature of seeds for establishment of plant and seed germination. Vigor value was observed using following formula (Bradbeer, 1988):

$$Vigor\ value = \left(\frac{a}{1} + \frac{b}{2} + \frac{c}{3} + \frac{d}{4} \ldots\ldots\ldots \frac{x}{n}\right) \times \frac{100}{S}$$

Where; a, b, c, d and x respectively represent the proportion seed germinated after 1, 2, 3 and n days of germination, S is the total number of germinated seeds. Mean germination time ($\bar{t}$) is the calculation of average time required for the germination of seed to take place. Mean germination time ($\bar{t}$) is the measure of rate and time-spread of germination. It was calculated by using following expression as described by Ranal et al. (2009):

$$\bar{t} = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

Where, ni= number of seeds germinated in the time i, k= last time of germination

Mean germination rate (MR) is the reciprocal of mean germination time. It is widely used to describe the speed of germination in relation with mean germination time ($\bar{t}$). Coefficient of variation of germination time is used to estimate the uniformity or variability of germination time. Coefficient of variation of germination time was calculated by Dorneles et al. (2005) using following relation.

$$CV = \frac{s_t}{\bar{t}} \times 100$$

Where; st= Standard deviation of germination time; $\bar{t}$=Mean germination time.

The analysis was carried out in replicates with weighted average sets for all the samples. A multiple comparison procedure of the treatment means was performed by Duncan's new multiple range test (Duncan, 1955). Significance of the differences was defined as ($P \leq 0.05$). Statistical analysis was carried out in IBM SPSS-16 software.

RESULTS AND DISCUSSION

Soaking kinetics and Peleg's constants: Moisture absorption in kidney bean was reported higher in distilled water-soaked grains followed by $MgCl_2$ (0.5%). From the absorption data as shown in figure 1, it was also observed that grains soaked in NaOH (0.1%) had lesser absorption of moisture which was slightly lower than $NaHCO_3$ (0.5%). There was lesser difference between the absorption curves of distilled water and $MgCl_2$ (0.5%). Moisture absorption in NaHCO3 (0.5%) soaked grains was higher during the early stages of soaking as compared to NaOH (0.1%) but slightly varied at the end of soaking process. Saturation point or equilibrium was attained in lesser time in distilled water-soaked group and required longer time in NaOH (0.1%) group.

Rate of absorption was observed in all treatment groups as shown in figure 2. Distilled water showed higher rate of absorption and thus attained saturation point at earliest followed by $MgCl_2$. Rate of hydration was lower in NaOH (0.1%) and $NaHCO_3$ (0.5%) soaking group. Kamkar et al. (2009) and Mendoza-Sánchez et al. (2016),

observed the negative impact of chemical stresses on the common bean due to structural and compositional changes to the seed coats. Sibian et al. (2016) concluded the fact that ions dissociated in soaking solution formed complexes with seed coat components and obstruct the process of active water uptake (Sibian et al. 2016).

Figure 1: Moisture absorption pattern in kidney bean (dry basis) in different soaking medium (NaOH-0.1%; $NaHCO_3$-0.5%; $MgCl_2$-0.5% and distilled water) at different time intervals.

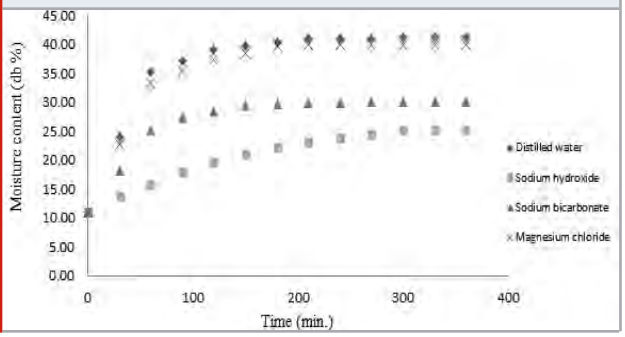
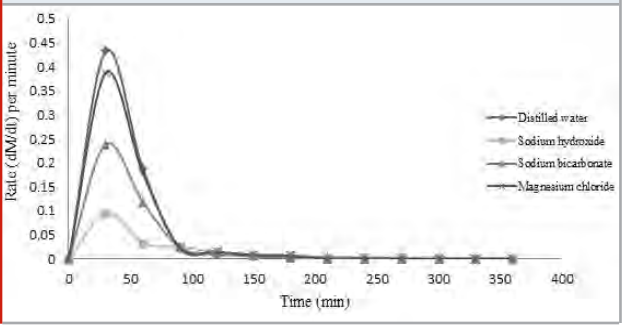


Figure 2: Rate of moisture absorption (dM/dt) during soaking in different soaking medium/solution (NaOH-0.1%; $NaHCO_3$-0.5%; $MgCl_2$-0.5% and distilled water) at different time intervals in kidney bean



Further confirmation of water uptake and hydration pattern was analyzed in Peleg's model equation. Peleg's constant K_1 & K_2 conveyed the information of soaking kinetics in term of two parameter empirical equation. Kidney bean grains are larger grains among other legumes and provided optimum surface area for water absorption but lower absorption rate due to the seed surface composition. Peleg's rate constant and capacity constant are inversely related to the rate of absorption and absorption capacities. Peleg's constants were in inverse relationship with hydration rate and capacity i.e. lower value of constants corresponds to higher water absorption rate and water absorption capacity (Sibian et al. 2013). Peleg's rate constant (K_1) in kidney bean soaking kinetics was lower in distilled water which could be justified due to higher initial water absorption rate. NaOH (0.1%) soaking solution gave the higher value of Peleg's rate constant, corresponding to its lower rate of absorption. Peleg's rate constant (K_1) for $MgCl_2$ (0.5%) was lower than distilled water-soaked grains followed by $NaHCO_3$ (0.5%).

Table 1. Summary of Peleg's Constant (K_1 & K_2) for kidney bean (cranberry bean) in different medium/soaking solutions.

	Rate Constant (K_1)	Capacity Constant (K_2)	r_2
Distilled water	1.57×10-2	2.94×10-2	0.98
NaOH-0.1%	1.46×10-3	4.29×10-2	0.98
$NaHCO_3$-0.5%	2.95×10-2	4.52×10-2	0.98
$MgCl_2$-0.5%	1.86×10-2	2.99×10-2	0.99

Peleg's capacity constant (K_2) was lower in distilled water and $Mgcl_2$ soaked grains followed by NaOH (0.1%) which was slightly lower than $NaHCO_3$ (0.5%) soaked kidney bean grains (Table 1). The mathematical expression of Peleg's constants, justified the hydration pattern as observed in figure 1 and 2. Non-ionic soaking medium like distilled water did not participated in complex formations with seed coat of kidney bean. If rapid uptake of water occurs it correspond to the fact that ions facilitate the movement of water without being affected by nature of seed wall components otherwise the interaction imparts negative effect on soaking behavior of grains as in present case of kidney bean. Variation in Peleg's constant due to chemicals were previously reported in pearl millet (Sibian et al. 2013) and chickpea (Sibian et al. 2016).

Dimensional Analysis: Dimensional analysis (Table 2) was done along the 3 geometric axis of seed grains to determine its length, width and thickness. Change in the length (Δl) varied insignificantly among all soaking solutions except $NaHCO_3$ (0.5%) soaked grains. Variation in width (Δw) was observed higher in distilled water and $MgCl_2$ (0.5%) soaked group (Δw=0.47±0.00 and 0.46±0.02 respectively). Value of variation in width observed in NaOH (0.1%) was 0.43±0.04 which was slightly higher than $NaHCO_3$ (0.5%) (Δw=0.38±0.01). Linear increase in the dimension was analyzed in all soaking groups during the soaking of grains. Bolaji et al. (2017) observed the similar pattern of increase in the dimensions of maize as a result of soaking (Bolaji et al. 2017).

Increase in thickness was uniform and ranged from 0.54±0.01 to 0.58±0.02. Spherecity (ϕ) and aspect ratio (Ra) did not vary significantly after soaking and showed uniformity among all groups. Shape of kidney bean plays an important role in the conformational changes during soaking. Volume of grains varied significantly after soaking and ranged from 54.99±0.20 to 60.57±0.21. Higher variation was observed in distilled water-soaked groups followed be $MgCl_2$ (0.5%), NaOH (0.1%) and

$NaHCO_3$ (0.5%). Surface area (S) for the grains after soaking varied from 1.18±0.04 to 1.24±0.02. Higher variation was observed in distilled water followed by both $MgCl_2$ (0.5%) and NaOH (0.1%), whereas $NaHCO_3$ (0.5%) showed least variation. Variation in dimensional attributes as a result of moisture uptake were also reported in cowpea (Yalcin, 2007) and popcorn kernel (Karababa, 2006).

Germination analysis: Computational data for germination analysis was generated using Microsoft excel sheet described by Ranal et al. (2009), to observe the relative frequency of germination after soaking under different chemical stresses. Soaking affected the total germination percentage of the seed. Higher germination percentage was observed in distilled water-soaked grains (78%), followed by $MgCl_2$ -0.5% (61%), $NaHCO_3$-0.5% (58%) and NaOH-0.1% (56%) as shown in table 3. The results are contrary to the observation made by Tobe et al. (2004), where halophytes were considered. Relative frequency of germination (*fi*) increases with the time period and is dependent on the type of soaking medium used (Tobe et al. 2004).

From the above calculated data, germination indices were prepared to observe the overall impact of soaking medium on germination. Relative to distilled water-soaked grains, all other germinated groups have shown slightly lower value of germination indices. Vigor value of $MgCl_2$ (0.5%) soaked kidney bean grains was higher and closely followed by distilled water and NaOH (0.1%) soaked grains. $NaHCO_3$ (0.5%) soaked kidney bean grains has lower vigor value. The mean germination time (MT) for all groups was observed same (78.72 hours) except NaOH (0.1%) soaked grains (MT=78 hours), which inferred that slightly lesser time was required for germination in NaOH (0.1%) soaked grains.

Table 2. Comparison of variation in dimensions of kidney bean (cranberry bean) after soaking in different soaking medium/solutions (NaOH-0.1%; $NaHCO_3$-0.5%; $MgCl_2$-0.5% and distilled water)

Characteristics	Material	Distilled water	NaOH (0.1%)	$NaHCO_3$ (0.5%)	$MgCl_2$ (0.5%)
Length (l)mm	Raw	15.84±0.02	15.81±0.01	15.83±0.03	15.82±0.03
	Soaked	16.15±0.04	16.11±0.01	16.10±0.01	16.13±0.02
Δl (mm)		0.31±0.03^{abc}	0.30±0.01^{cab}	0.27±0.04^{d}	0.31±0.04^{bca}
Width (w)mm	Raw	7.35±0.01	7.32±0.02	7.34±0.02	7.33±0.02
	Soaked	7.81±0.01	7.75±0.04	7.72±0.04	7.79±0.01
Δw (mm)		0.47±0.00^{ab}	0.43±0.04^{c}	0.38±0.01^{d}	0.46±0.02^{ba}
Thickness (t)mm	Raw	4.47±0.02	4.55±0.01	4.52±0.01	4.56±0.04
	Soaked	5.14±0.01	5.09±0.01	5.10±0.01	5.12±0.01
Δt (mm)		0.57±0.02^{ba}	0.54±0.01^{d}	0.58±0.02^{ab}	0.56±0.05^{cb}
Spherecity (ϕ)	Raw	0.51±0.00	0.51±0.00	0.51±0.00	0.51±0.00
	Soaked	0.54±0.00	0.53±0.00	0.53±0.00	0.54±0.00
Δϕ		0.03±0.00^{ab}	0.02±0.00^{cd}	0.02±0.00^{dc}	0.03±0.00^{ba}
Aspect ratio (Ra)	Raw	0.46±0.00	0.46±0.00	0.46±0.00	0.46±0.00
	Soaked	0.48±0.00	0.48±0.00	0.48±0.00	0.48±0.00
ΔRa		0.02±0.00^{abcd}	0.02±0.00^{bacd}	0.02±0.00^{cabd}	0.02±0.00^{dacb}
Volume (V)mm^3	Raw	294.40±0.23	291.44±0.34	291.38±0.21	292.83±0.51
	Soaked	354.97±0.20	347.78±0.46	346.38±0.21	352.23±0.45
ΔV (mm3)		60.57±0.21^{ab}	56.33±0.41^{c}	54.99±0.20^{d}	59.40±0.47^{ba}
Surface area (S) mm^2	Raw	11.14±0.02	11.08±0.03	11.05±0.01	11.11±0.06
	Soaked	12.38±0.02	12.26±0.01	12.24±0.05	12.33±0.04
ΔS (mm^2)		1.24±0.02^{a}	1.22±0.03^{bc}	1.18±0.04^{d}	1.22±0.05^{cb}

Values are expressed as mean ± standard deviation. Means having different letters within the same row differ significantly at $p \leq 0.05$ ($n = 3$). Δ denotes the differences in the respective dimensional parameter's values.

Mean germination rate $\bar{\upsilon}$ was observed same in all cases ($\bar{\upsilon}$=0.30) with non-significant variation. Coefficient of variation of germination time (CV) was observed higher in $MgCl_2$ (0.5%) soaked grains followed by distilled water, NaOH (0.1%) and $NaHCO_3$ (0.5%). According to the seed germination studies conducted by Pereira and Santana (2013) on different species of same family, the increased coefficient of variation is not capable of predicting the heterogeneity of variance, but the data can be used to monitor the germination capability of seed

grains. In lab seed germination analysis also provide data to the various food production sections where seed germination is required viz breweries. Research has also shown the utilization of germinated grains in composite flours to enhance nutrition index of bakery products (Sibian et al. 2020).

Table 3. Analysis of germination data and germination indices of kidney bean after soaking in different soaking medium/solutions (NaOH-0.1%; NaHCO3-0.5%; MgCl2-0.5% and distilled water).

		NaOH (0.1%)				
Time in days	No. of seed germinated (n)	n*t	t-MT	$(t-MT)^2$	$n(t-MT)^2$	(*f*i)
1	2	2	-1	1	2	0.0204
2	7	14	0	0	0	0.0714
3	22	66	1	1	22	0.224
4	25	100	2	4	100	0.2551
Germination Indices						
G (%)	V	MT	(υ)	CV		
56	34.08	78	0.31	0.46		
		$NaHCO_3$ (0.5%)				
1	2	2	-1	1	2	0.0204
2	6	12	0	0	0	0.0612
3	24	72	1	1	24	0.2449
4	26	104	2	4	104	0.2653
Germination Indices						
G (%)	V	MT	(υ)	CV		
58	33.62	78.72	0.31	0.46		
		$MgCl_2$ (0.5%)				
1	4	4	-1	1	4	0.0408
2	6	12	0	0	0	0.0612
3	20	60	1	1	20	0.2041
4	31	124	2	4	124	0.3163
Germination Indices						
G (%)	V	MT	(υ)	CV		
61	35.11	78.72	0.31	0.48		
Distilled water						
1	5	5	-1	1	5	0.0510
2	4	8	0	0	0	0.0408
3	33	99	1	1	33	0.3367
4	36	144	2	4	144	0.3673
Germination Indices						
G (%)	V	MT	(υ)	CV		
78	34.62	78.72	0.30	0.47		

Where t= time between sowing and the day of observation G=germination percentage, V=Vigor value, MT=mean germination time in hours, υ=Mean germination rate, CV=Coefficient of variation of germination

CONCLUSION

Different chemical stresses exhibit different absorption and germination characteristics. The extent of soaking and rate of absorption can easily be estimated from Peleg's mathematical equation. Ionic strength of solution has proven as detrimental factor to be considered during soaking. Soaking caused changes in the overall dimension of grains. Germination indices provided the detailed insight of germination process. It was also concluded from the obtained data, that germination indices of grains are independent from soaking kinetics. While the seed enables to attain saturation point by the uptake of optimum moisture, the seed will be

enabled to germinate despite of the salt stress. However, germination percentage is greatly affected by the chemical stress. Mean germination rate and coefficient of variation of germination were found independent from germination percentage and mean germination time. It was observed that despite having significant difference in the germination percentage and vigor of different groups, there was similarity in some germination indices, which stated the importance of germination index over germination percentage and salt stress management in agriculture.

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Nutritional Communication

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Determining the Role of *Morinda citrifolia* and *Stevia rebaudiana* as Nutritional Enhancers

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ABSTRACT

Morinda citrifolia and *Stevia rebaudiana* are the buzz words from plant kingdom for the diabetic community. As known to all, diabetic people are devoid of the privilege of consuming sweets or any food sweet in taste. Though, there are several special foods available for diabetics in market, the jams or spreads is most limited. Jam being most favoured spread for people of all ages, especially on the top of frequently consumed breads, it becomes obligatory to enhance its nutritional parameters. Hence, as a pilot initiative this study aims to develop value added jam consumable by diabetics. Jam was developed by incorporating noni fruit pulp (*Morinda citrifolia*) as a major ingredient and stevia (*Stevia rebaudiana*) leaves extract as a source of sweetness. The developed value added jam was subjected to physical, organoleptic and nutritional evaluation. The brix value of the value added jam was 68.50, pH 3.3 and moisture was 29.73%, which met the set standards. The organoleptic evaluation score high in all aspects like appearance, texture, flavour, mouthfeel and overall acceptability. The valued added jam, with noni fruit pulp and stevia powder incorporated was found low in calories and carbohydrates and rich in fibre, calcium, magnesium and potassium. It was observed that the resultant product was rich in micronutrients with enhanced taste and can be considered as an apt complimentary food for diabetics and persons with liver disorders.

KEY WORDS: DIABETES MELLITUS, JAM, MORINDA CITRIFOLIA, STEVIA REBAUDIANA, VALUE ADDITION.

INTRODUCTION

The main components of this study are *Morinda citrifolia* and *Stevia rebaudiana*. *Morinda citrifolia* commonly known as Indian mulberry or noni fruit has been cultivated since 400 A.D. Therapeutically, noni is used as a medicine due to its antimicrobial and antioxidant properties (Duduku Krishnaiah et.al, 2015). Some of the medicinal and functional properties of noni includes wound healing, promotes a healthy inflammatory response, reinforces the body's ability to fight infection, helps maintain healthy blood sugar levels and strengthens cells at the micro level (Aline Carla Inada et.al, 2017). Noni juice has found to enhance neural-immune interactions and cell survival pathways while inhibiting inflammatory processes in age-associated diseases (Jahidul Islam, 2019). Although noni is power packed with phytonutrients or nutraceutical compounds like alizarin an anthroquinone, epigallocatechin gallate,

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limonene, terpenes like beta-carotene and fibre its use is scarce. However, it still requires more research aimed at standardization to raise the quality of products that are already in the market, such as Noni fruit juice, and to elucidate the real potential of this plant. (Edipo.S. Almedia et. al, 2019).

Stevia rebaudiana generally known as sweet tulsi, is composed of several natural, heat-stable steviol glycosides (Margaret Ashwell, 2015) with different intensities of sweetness and flavour profiles which differ from each other and vary according to concentration and environment of growth. Due to its low calorific value and intense sweetness stevia will prove as an effective alternate to table sugar. Unlikely, the artificial sweeteners like aspartame have gained popularity as a sugar substitute for diabetes mellitus than stevia. Stevia has proved to exhibit bactericidal activity, possess antioxidant properties, lowers blood sugar levels and controls blood pressure (Rojas E et. al, 2018). In addition to be used as intense sweetener, Stevia helps in preparation of functional and medicinal foods that augment health status of masses (Muhammad Farhan Jahangir Chughtai et. al, 2020).

Jam, a versatile food product used globally is high in calories. Jam includes fruits in its preparation and hence seems to be a healthier food product than jellies and preserves (Kevin Farrell, 2020). As the diabetic people are deprived of relishing the taste of jam, the development of value added jam by incorporating noni fruit pulp and stevia will benefit the needy diabetics.

MATERIAL AND METHODS

Procurement and Processing of Raw Material: Different ingredients for the development of jam like noni fruit, stevia powder and pectin powder were procured from the local departmental store in Salem district, Tamil Nadu. Processing of Noni Fruit: Noni fruits were purchased and checked for any infestation or damage. Noni fruits free from damage were then kept to ripen. The noni fruits were mashed and ground, after peeling skin and removing seeds, to make pulp.

Physical and Functional Characteristics of Noni Fruit: The physical characteristics of noni fruit such as mass, length, circumference, density, seed size and functional traits such as juice recovery and mass of the pulp were assessed.

Formulation of Noni Fruit Pulp and Stevia Powder Incorporated Value Added Jam: Pulp from the fruit were processed into fruit jam according to the FAO guidelines (Mircea Enachescu Dauthy, FAO, 1995). Pectin from different sources was tested for gelling capacity needed to produce acceptable jam. The sources of pectin were the natural pectin present in the fruit itself and commercial pectin powder.

The list of ingredients and their level of incorporation for preparing the product are given in Table 1. 100gms of noni fruit pulp and 10gms stevia powder was placed in a stainless steel kettle and heated to about 110°C under constant stirring and was turned low. 0.2 gms of pectin powder was mixed with another 5gms of stevia powder, and then added into the fruit pulp and stirred constantly to prevent the pectin from coagulating. When the pectin dissolved completely, the remaining 5gms of stevia powder was added and dissolved completely in the mixture. The heat was then increased and the jam mixture was stirred constantly, until vigorous boiling started. Near the finishing point approximately 221°C, lemon juice was used to justify the customary acidity. For the control jam strawberry fruit pulp was used as, strawberry fruit jam was considered as most favourite through survey. Same methodology with 100 gms of strawberry fruit pulp and 20gms of sugar was followed for the preparation of control jam.

Table 1. Ingredients in the Preparation of Control Jam and Value Added Jam

Ingredients	Level of Incorporation	
	Control Jam	Noni Fruit Pulp and Stevia Incorporated Value Added Jam
Noni fruit pulp (gms)	-	100
Strawberry fruit pulp	100	-
Stevia powder (gms)	-	20
Sugar (gms)	20	-
Pectin powder (gms)	0.2	0.2
Lemon juice (ml)	10	10

RESULTS AND DISCUSSION

The findings of the study are presented below.

Physical and Functional Characteristics of Noni Fruit and Pulp:

Fruits in different maturation stages may be found in the same shrub to tree (Carrillo-Lopez and Yahia, 2011). The fruits can be harvested at different stages of maturation, which will continue to ripen naturally. The ripening process of the fruit comprises five phases that correspond to the tonality and hardness of the fruit (Chan-Blanco et al. 2006). Noni fruit has a shelf-life of 5 to 7 days at an ambient temperature between 25 and 30°C and relative humidity between 70 and 75% (Singh DR et al. 2007). The noni fruit has brown colored seeds (3 to 9 mm long), housed in groups of four inside numerous reddish-brown, triangular-shaped grooves (Dittmar, 1993).When dried in the air noni seed is lightweight, weighing about a quarter of gram, and its coating is made up of extremely resistant layers of cellulose fibers. Its interior is composed of bulbous ovoid chamber where the embryo is housed. The embryo is quite small (few millimetres), flat and oily (Nelson, 2005). It is evident from table 2 that the physical and functional traits of

noni fruit and pulp used for the study complied with the values reported by various researchers.

Functional Properties of the Control Jam and Developed Value Added Jam: The Physical and functional properties of noni fruit pulp and stevia powder incorporated value added jam was evaluated. The parameter such as brix, pH and moisture were studied and the results are presented in the table 3.

Brix scale is commonly used for total sugar content measurement for any substance. The range from 40 to 70o brix is required for fruit jam to be acceptable (Azam ali, 2007). Up to 70o brix, jam doesn't require pasteurization, a process that inhibits microbial growth, and extends the shelf life of the product. Table 3 shows that the developed value added jam with noni fruit pulp and stevia showed a brix value of 68.50.The pH was recorded as 3.3, which reflects that the developed product was acidic in nature. Studies show that the property of forming a viscous semi-solid gel is achieved at a pH of 3.2 to 3.4. Moisture content was 29.73%. The physical and functional characteristics of the developed value added jam met the same standards as that of the control jam (Hui. H.Y. 2006, T. M. Rababah et al, 2014). All these factors had been favourable for the development of a high quality jam.

Table 2. Physical and Functional Characteristics of Noni Fruit and Pulp

Physical and Functional Parameters	Measured Values of Noni Fruit and Pulp Used for the Study	Nelson (2003-2005)	Singh et al. (2007)	Carrilo-Lopez and Yahia (2011)
Mass (g)	151.20	-	147.9	50 to 300
Average length (cm)	8.6	14	9.8	4 to 10
Circumference (cm)	4.8	8	5.26	3 to 4
Density (g/cm^3)	-	-	1.13	-
Juice recovery (%)	46.90	≅50	38.95 to 48.50	-
Relative mass of pulp (%)	45.71	-	44.76 to 46.72	-
Seed size (mm)	3.3	4 to 9	3 to 5	4 to 6

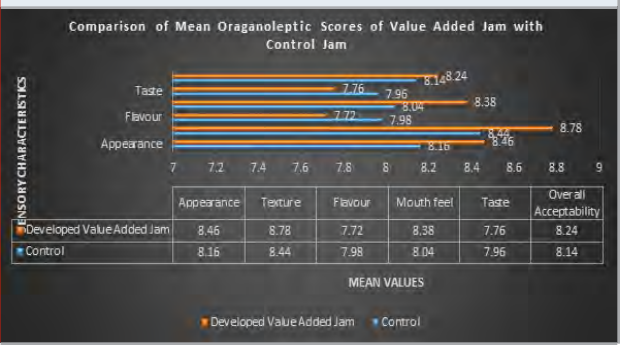
Organoleptic Evaluation of the Control Jam and Value Added Jam: 9 point hedonic rating scale method was adopted to estimate the organoleptic acceptance of the developed product. Totally 50 semi trained people were used for organoleptic analysis. The sensory parameters like appearance, texture, flavour, mouthfeel, taste and overall acceptability of the value added jam developed by incorporating noni fruit pulp and stevia and control strawberry jam were assessed. Figure 1 depicts the comparative results of sensory parameters between the value added jam and control jam. Except the taste all the other sensory parameters were high in the developed value added jam. The overall acceptability was also high for the developed value added jam. After organoleptic evaluation the products were further subjected to nutritional evaluation.

Table 3. Functional Characteristics of Value Added Jam

S.No	Functional characteristics	Control Strawberry Jam	Developed Value Added Jam
1.	Brix	680	68.50
2.	pH	3.4	3.3
3.	Moisture	22.3%	29.73%

Nutritional Value of the Control Jam and the Value Added Jam: The purpose of this study is to develop a jam that can be consumed by the diabetic population as well to enhance the nutritional value of the jam. As a means of value addition the noni fruit and stevia powder, a rarely used plant species have been utilised for the development of jam. Since it has passed the organoleptic evaluation, nutritional analysis was carried out. The nutrient content of the control jam as well as the value added jam is projected in Table 4.

Figure 1: Comparison of Mean Oraganoleptic Scores of Value Added Jam with Control Jam



The noni fruit pulp and stevia powder incorporated value added jam provided 118.05 Kcal of energy, 29.2g of carbohydrate, 0.2g and 0.05 g of protein respectively. 16.65 gms of fibre was present. It is also understood that the value added jam contained 1.1mg of Vitamin C. Calcium, sodium, potassium and magnesium were

present at a level of 23.4mg, 14.6mg, 110mg and 10.2mg respectively for 100gms of accepted variation of value added jam. The commercialisation of what once was a homemade jar of goodness is now termed unhealthy because it contains more sugar and less fruit. Sometimes it is only the essence of the fruit or fruit juice and a whole lot of additives and preservatives, while our value added jam prepared with noni pulp and stevia proves vice versa (Shanthini Rajkumar, 2020).

Table 4. Comparison of Nutrient Composition of the Value Added Jam with Control Jam

Nutritional Parameters	Control Jam	Value Added Jam
Ash (%)	0.25	3.3
Energy (kcal)	280	118.05
Carbohydrate (g)	68.85	29.2
Protein (g)	0.35	0.2
Fat (g)	0.05	0.05
Fibre (g)	1	16.65
Vitamin C (mg)	6	1.1
Calcium (mg)	20	23.4
Sodium (mg)	30	14.6
Potassium (mg)	75	110
Magnesium (mg)	0.04	10.2

On comparison with the strawberry control jam, the valued added jam, with noni fruit pulp and stevia powder incorporated was found low in calories and carbohydrates. Vitamin C and sodium was more in the control jam which may be attributed to the use of strawberry pulp rich in these nutrients. Fibre, Calcium, Magnesium and Potassium were found in higher proportion in value added jam compared to the control. The presence of noni fruit in the jam would also help to alleviate stress and oxidative inflammation (Xiaobing Yang et. al, 2020). Apart from prescribing for diabetics, this jam can also be recommended for persons with liver injury as noni fruit showed higher antioxidant capacities against acute alcoholic-induced liver injury (Min Guo et. al, 2020).

CONCLUSION

It can be concluded the nutritional parameters of jam prepared using noni fruit pulp and stevia powder is superior compared to control jam. The value added jam prepared will prove effective and valuable for diabetic patients as it is low in carbohydrates and calories. Noni has a favourable effect on liver disorders. Noni is are rarely used as a whole fruit or processed because of its unpleasant taste. Noni juice is the product commonly available, hence the food industries can be encouraged to use noni fruit in various recipes or menus, as a means of value addition. Although stevia has been substantiated as a substitute for sugars or commercial artificial sweeteners, the promotion on the usage of stevia is insufficient. It is endorsed that further cognizance is vital, regarding the ubiquity and prominence of noni as an anti-diabetic fruit and stevia as an alternate sweetener. As noni is high in potassium, people with renal diseases should avoid. Research studies with human clinical trials is recommended to promote the potentiality of noni fruit for non-communicable diseases.

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Development of Value-Added Nutritious Crackers Incorporated with Corn Silk Powder

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ABSTRACT

Recently, consumer demand for healthy snacks has increased. Crackers are popular healthy snacks with high potential to enhance the nutritional value by incorporating naturally available ingredients. Corn silks are a bundle of silky, long and yellowish strands which could be seen on top of both baby corn and corn fruit. Corn silk is a byproduct of corn with high nutritional value and antioxidant property, hence dried corn silk powder incorporated food products can be considered good for health and suitable for all age groups. Corn silk is used as a medicinal herb by practitioners of traditional medicine all over the world and is documented as a well-accepted traditional medicine in treating non-communicable diseases like Diabetes mellitus, cardiovascular diseases, kidney diseases and cancer. Incorporation of corn silk powder in food products results in increasing protein, fibre, minerals, cooking yield, moisture and fat retention while decreasing fat content. This study is aimed with the objective of formulating crackers by incorporating dried corn silk powder in different variations. The developed crackers were subjected to analysis of physical characteristics, sensory evaluation and the accepted variation was subjected to analyze nutritional compositions by using standard procedures. It was observed that the corn silk powder incorporated cracker was significantly rich in protein, fibre, vitamin C, calcium and magnesium compared to control crackers. Besides these properties, corn silk powder contains flavonoids. The antioxidant activity of flavones is associated with the prevention of cancer and cardiovascular diseases which is also promising. It was evident that the prepared crackers were more economical and affordable when compared with commercial soup mixes available in the market..

KEY WORDS: ANTIOXIDANT PROPERTY, DRIED CORN SILK POWDER, NUTRITIONAL VALUE, VALUE- ADDED FOOD PRODUCTS.

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INTRODUCTION

Globally, Corn, is the most widely grown and third most important cereal crop next to wheat and rice. Corn Silk, the silky hair-like structures protruding from the tip of the ear of corn, is essentially the elongated stigma of the female flower of Corn. Although often discarded as agricultural waste or byproduct of Corn cultivation, Corn Silk has also been used as traditional remedies in different parts of the world for the treatment of kidney stones, diuretic, bloating, liver problems, chronic cystitis, urethritis, and prostatitis, (and other prostate disorders). urinary infections and obesity (Liu et al. 2011). Corn Silk contains 9–22% protein content on a dry mass basis, depending on its stage of maturity and corn variety (Haslina et al. 2017). Corn silk has phenolic compounds, particularly flavonoids. It also consists of proteins, vitamins, carbohydrates, calcium, potassium, magnesium and sodium salts, volatiles oils and steroids such as sitosterol, stigmasterol, alkaloids, and saponins. Corn silk is also said to be an excellent source of vitamin K, which has been known to slow bleeding. Corn silk also helps in reducing blood pressure (Vijitha et al. 2017).

Food-derived bioactive peptides received growing attention from the international research community over the last two decades. These multifunctional peptides are used for the prevention of cardiovascular disease. Antioxidant peptides, both in the form of protein hydrolysates as a mixture and in the form of pure individual peptides, are recognized as potent, natural alternatives to synthetic antioxidants for application as food additives. Such peptides are also potential for the future development of functional food ingredients and therapeutic agents (Lammi et al. 2019). Crackers are snack products enjoyed by people of all age groups. Corn silk incorporated cracker is rich in calories, protein, fibre and minerals. The value added by incorporating corn silk powder will enhance the nutritional quality apart from quenching the craving for a snack. Hence, in this, it was proposed to exploit corn silk powder as a means of value addition in the favourite novel product, crackers (Wang et al. 2019).

MATERIAL AND METHODS

The fresh corn silk (Zea mays) was collected in the local market Salem and stored at room temperature. Processing of corn silk into powder can cause changes in the chemical characteristics of corn silk powder. The level of this change depends on the drying method used to optimize the drying process and maintain the quality of the dried product. The drying method most commonly used in the food industry is the conventional oven-drying method using hot air which works by evaporating water from the material. The corn silk was dried in hot air oven dryer for two and half hours at 60˚C to make it easy for made it into powder (Roshli et al. 2011). The corn silk powder stored in the refrigerator in an airtight container at 4˚C (Haslina et al. 2017).

Take processed dried corn silk powder (in different variations) to the required amount. Add whole wheat flour and also add milk powder, olive oil, baking powder, sugar powder, salt according to the quantity needed. Mix it well and make dough with a thick consistency and leave it for 5 minutes. Cut the dough into required pieces with a suitable shape. All the three different variations of developed crackers were baked in an oven at 170 ˚C for 15 minutes and stored in a sealed plastic pouch. After the preparation of crackers, the physiochemical characteristics, sensory evaluation and nutritional compositions of the developed crackers were analyzed.

RESULTS AND DISCUSSION

Development of crackers incorporating corn silk powder: To formulate crackers, blend corn silk powder in the required amount, whole wheat flour, milk powder, olive oil, baking powder, sugar powder, salt in required quantities to suit the different variations of crackers to be developed. The composition of ingredients used for developing crackers of different variations is shown in Table – 1

Table 1. Composition of ingredients for corn silk powder incorporated crackers

Ingredients	Control	Variation 1	Variation 2	Variation 3
Whole wheat flour	100g	90g	80g	70g
Corn silk powder	-	10g	20g	30g
Sugar powder	30g	20g	20g	20g
Milk powder	10g	10g		10g
Olive oil	15ml	15ml	15ml	15ml
Baking powder	1.5g	1.5g	1.5g	1.5g
Salt	1.5g	1.5g	1.5g	1.5g

Physical characteristics of the developed crackers: Height, weight, diameter and thickness of the control crackers and dried corn silk powder incorporated crackers were assessed (Baljeet et al. 2010). The Physical characteristics of the developed crackers are presented in Table -2.

Table 2. Physical characteristics of the developed crackers

Variations	Height (cm)	Weight (kg)	Diameter (cm)	Thickness (cm)
Control crackers	5.65	24.74	5.64	0.30
Variation 1	5.58	21.07	5.83	0.33
Variation 2	5.67	22.34	5.67	0.35
Variation 3	5.45	24.58	5.58	0.28

Compared to control crackers, crackers formulated with dried corn silk powder showed an increase in diameter and thickness. Corn silk powder added crackers had lower weight and there is a slight difference in height compared to control crackers.

Sensory evaluation of the developed corn silk powder incorporated crackers: The most widely used scale for measuring food acceptability through senses is the 9-point hedonic scale. This scale was used for evaluating the sensory properties of the crackers. Three variations of the crackers were developed by the incorporation of corn silk powder. Sensory evaluation was carried out by panel members, semi-trained consumers consisting of students and staff of the Department of Nutrition and Dietetics, Periyar University, Salem, Tamil Nadu, India. They evaluated crackers with respect to different sensory parameters, namely colour, texture, mouthfeel, taste, flavour, crispness and overall acceptability on a 9-point hedonic scale. Significance was established at $P \leq 0.05$ using statistics outlined below.

Table 3. Statistical analysis of sensory evaluation of the developed crackers

Samples	Colour	Texture	Flavour	Mouthfeel	Taste	Crispiness	OAC
Control	4.65 ± 1.23^{c}	5.77 ± 0.14^{a}	5.62 ± 0.28^{b}	5.12 ± 0.27^{ab}	3.40 ± 1.75^{a}	6.70 ± 1.81^{a}	5.21 ± 1.18^{ab}
Variation 1	7.10 ± 1.62^{ab}	7.40 ± 1.19^{a}	8.61 ± 1.32^{ab}	9.40 ± 1.20^{bc}	8.79 ± 1.26^{c}	7.26 ± 1.22^{ab}	8.40 ± 1.33^{a}
Variation 2	4.72 ± 1.11^{ab}	5.81 ± 1.1^{c}	6.40 ± 1.41^{ab}	3.98 ± 1.81^{ac}	5.61 ± 1.84^{ac}	4.81 ± 1.14^{a}	4.51 ± 1.72^{ab}
Variation 3	3.89 ± 0.84^{a}	4.20 ± 1.18^{a}	3.45 ± 1.82^{c}	2.51 ± 1.19^{bc}	4.15 ± 1.19^{a}	3.47 ± 0.31^{a}	4.23 ± 0.24^{ab}

Values are Mean ± SD of triplicate determination. Samples with different superscripts within the same column were significantly ($p \leq 0.05$) different.

The results of the above table revealed that the mean score obtained for the colour of V1 and V2 were found to be maximum score (7.10 ± 1.62^{ab} and 4.72 ± 1.11^{ab}) than control and V3. Mean score of texture was high (7.40 ± 1.19^{a}) in V1 compared to control and other variations. The results revealed that the mean score obtained for the flavour of V1 found to be superior (8.61 ± 1.32^{ab}) compared to control and other variations.

The decrease in the addition of corn silk powder had a satisfactory effect on flavour and mouthfeel of crackers. Variation I had the maximum mean scores of 9.40 ± 1.20^{bc} for mouthfeel, thus confirming that addition of corn silk powder at 10gm is more acceptable whereas V3 and V2 had scored lower than V1 due to the less addition of other ingredients which affected the mouthfeel of the end product.

Table 4. Nutritional composition of accepted variation of the crackers

S. No	Nutrients	Control crackers	Corn silk powder incorporated crackers (variation-1)	Deficient or excess
1.	Moisture (%)	2.07	5.67	+3.6
2.	Energy (Kcals)	384	398	+14
3.	Carbohydrate (g)	60.14	76.5	+16.36
4.	Protein (g)	1.1	7.90	+6.8
5.	Fat (g)	5.74	4.61	-1.13
6.	Fibre (g)	0.90	3.25	+2.35
7.	Vitamin C(mg)	-	2.6	+2.6
8.	Magnesium (mg)	0.24	1.5	+1.26
9.	Calcium (mg)	-	0.26	+0.26
10.	Flavonoids	-	++	-

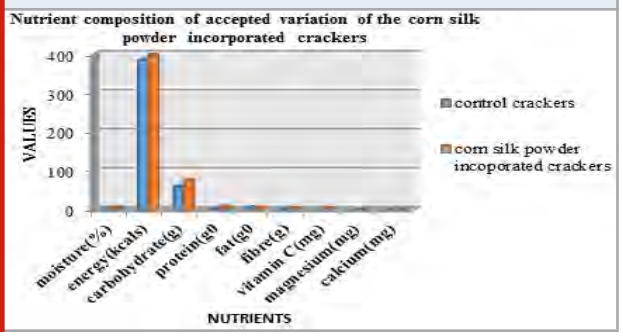
Mean taste scores of control crackers prepared with whole wheat flour, milk powder, olive oil, baking soda, sugar was low (3.40 ± 1.75a) compared with variation 1, 2 and 3 (8.79 ± 1.26c, 5.61 ± 1.84^{ac} and 4.15 ± 1.19^{a}) respectively. The crispiness of Variation 3 was low (3.47 ± 0.31^{a}) compared to variation 1 (7.26 ± 1.22^{ab}). As an overall result, the overall acceptability of the corn silk incorporated (10gm) cracker of variation 1 was high (8.40 ± 1.23^{a}) on a hedonic scale. Results on Duncan Multiple Range test showed that there was a significant difference ($p < 0.05$) between

control and the different variations of crackers on colour, texture, flavour, mouthfeel, taste, crispiness, and overall acceptability. Consumers are the judges of a product's fate and welfare in the market as their preference is of vital significance. Therefore, specific sensory properties of a product, along with its composition, may comprise a key for its uniqueness and support (Vieira et al. 2008).

Nutritional composition of accepted variation of the crackers: Based on the physical characteristics and sensory evaluation of the developed crackers, 10% of corn silk powder incorporated cracker (Variation 1) was highly accepted. Hence the nutrient analysis was done for variation 1. The nutrient analyses of control crackers and crackers formulated with 10% of dried corn silk powder are shown in table -4.

The Moisture content of the corn silk powder incorporated crackers ranged from 5.67%, which was higher than the control crackers because cornsilk contains higher moisture content. There was a slight change in the calorie content between the developed product and control. The developed product showed 7.90g of protein, whereas control crackers had 1.1g. The addition of corn silk powder to cracker formulation increased the protein content of the tested products. The carbohydrate content of the developed product showed 76.5g, which was higher than the control crackers. The magnesium content of the corn silk powder incorporated crackers had 1.5mg/100g. Calcium level present in the 10% incorporation of corn silk powder incorporated crackers was 0.26mg, whereas the control cracker does not have any calcium content which showed that corn silk powder incorporated crackers improved calcium level. Besides these nutritional properties, corn silk powder added crackers contain flavonoids. The antioxidant activity of flavones was associated with the prevention of cancer and coronary heart diseases.

Figure 1: Nutritional composition of accepted variation of the crackers



Cost calculation of the developed food products: The cost calculation for the production of 100gm of developed crackers revealed that the total production cost (100g) was Rs.55.00 by incorporating corn silk, whole wheat flour, milk powder, olive oil, sugar and baking powder. It was evident that the prepared crackers were more economical and affordable when compared with commercial crackers available in the markets.

CONCLUSION

In conclusion, corn silk could be considered as a good source of nutritional composition and antioxidant activity. Incorporation of corn silk powder resulted in increased protein, fibre, vitamin C, calcium and magnesium in the crackers. Crackers with 10% corn silk added were highly acceptable by the consumers. This novel corn silk for incorporation in crackers could permit a reduction of formulation cost without affecting sensory attributes of the developed product to which the consumer is familiarized. This byproduct is also being utilized as hypo glycemic agent, diuretic agent, antioxidant and other therapeutic functionalities. The benefits of corn silk in improving other pharma-nutritional functionalities, including prebiotics potential, can be recommended for future studies.

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A Minireview on Antimicrobial Peptides of Goats and their Role in Host Defense

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ABSTRACT

Antimicrobial peptides play an important role in host defense and they are nearly present in all forms of life. Domestic goat (*Capra hircus*) also known as poor's man cow is a backbone to lower income group people of India. Goats are reared generally for meat and milk purposes. Goat has also been found to express different types of antimicrobial peptides like defensins, cathelicidins, having broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, and fungi. Some of them may have cytotoxic effects also. These antimicrobial peptides may also act an immunomodulators. This review briefly describes antimicrobial peptides identified from goat and their potential act as an immunomodulators role in host defense.

KEY WORDS: ANTIMICROBIAL PEPTIDES, *CAPRA HIRCUS*, DEFENSINS, CATHELICIDINS, S100A8, HEPCIDIN.

INTRODUCTION

Goat is one of the oldest domesticated animals, popularly known as mortgage lifters of India along with sheep. India occupies second position in terms of goat population and first position in goat milk production. Goat meat known as Chevon, is most preferred and widely consumed meat in the country and constitutes about 37% of total meat production. Goat milk possesses many advantages over cow milk as a nutritional source for infants and children (Kumar and Sharma, 2016). Since ages Goats have been poor people's most reliable livelihood resource. India has 34 registered breeds of Goat (Hegde, 2020).

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Goats are resistant to many diseases and they have ability to survive in harsher conditions compared to other ruminants. Antimicrobial Peptides (AMPs) are evolutionary conserved in the genome and produced by all life forms, from prokaryotes to humans (Hancock and Diamond, 2000). In animals, AMPs are believed to be the first line of the innate immune defense against bacteria, fungi and viruses (Zasloff, 2002). They are widely distributed in animal tissues and cells that are exposed to invading organisms. The first mammalian peptides, MCP-1 & 2 were isolated from rabbit macrophages (Selsted et al., 1983). AMPs are produced by polymorphonuclear leukocytes, macrophages and lymphocytes of the immune system (Radek and Gallo, 2007) and by all epithelial cells in response to the direct contact with microbes. These peptides exhibit direct anti-microbial activity as well as chemotactic and regulatory functions and plays an important role in immunity.

At least five genes present in goat genome have been identified which encodes for these antimicrobial peptides (Zanetti, 2005). The antimicrobial peptides are nowadays used as a medicated feed additive in the rations of

ruminants, swine and poultry. A combination of recombinant porcine β-defensin-1 and a fly antibacterial peptide in a ratio of 1:1 was used as a medicated feed additive for juvenile goats leading to increased body weight, average daily weight gain, enzymatic activity, influence on ruminal fermentation function and higher rumen microorganism diversity indices (Liu et al., 2017). These peptides have been described to be effective against many Gram-negative and Gram-positive bacteria, fungi, protozoa, viruses as well as cancer cells. Bioactive peptides from goat milk casein hydrolysates ameliorated insulin resistance in HepG2 cells that had been treated with high glucose (Gong et al., 2020).

Antibacterial activity of goat urinary cationic antimicrobial proteins against bacterial strains of *Staphylococcus aureus* and *E. coli* has been demonstrated (Tomar et al., 2018). Researchers across the globe have been able to identify antimicrobial peptides from the native goat which comprise mainly the Defensins and Cathelicidins. Antimicrobial peptides from goats and its source tissue has been presented in Table-1. The three dimensional structure of goat antimicrobial peptides has been depicted in Figure-1. Antimicrobial peptides have been shown to have immunomodulatory properties that includes gene expression, chemotaxis, wound healing properties and cytokine release. These peptides suppress the toll like receptors (TRL) signalling and tumor necrosis factor-α (Haversen et al., 2002; Davidson et al., 2004). The antibacterial cationic peptides are at an early stage of drug development.

However, the development of AMPs as potential therapeutics is hindered by several challenges like low specificity, high manufacturing cost, and potential toxicity to animal cells (Bahar and Ren, 2013). These peptides also have least ability to develop resistance due to the ability of these peptides (AMPs) for attacking multiple low targets rather than one defined, high target, characteristic for conventional antibiotics (Mahlapuu et al., 2016). Many statistical and computational algorithms like support vector machines (SVM), hidden Markov model, artificial neural networks (ANN) with cheminformatics approaches is being used for the development of novel antimicrobial peptides (Divyashree et al., 2020). Various *in-silico* approach is being tried for designing novel peptides (Farcas et al., 2020). Development of antimicrobial peptides to use as a dietary supplement for human for therapeutic purposes against pathogens has been described (Bakare et al., 2020). The use of antimicrobial peptides in the age of resistance provides immense opportunities in dealing with the multidrug-resistant pathogens (Magana et al., 2020). This review has been briefly summarized considering the role of caprine antimicrobial peptides in host defense.

Defensins: Defensins are small (29-45 amino acid residues) cationic antimicrobial peptides with β-sheet structures that are stabilized by three intramolecular disulfide bonds (Lehrer and Ganz, 1996). Three different types of defensins namely α-, β- and θ- have been identified till date, most common being the β-defensins. θ–defensin have been isolated only in rhesus monkey leukocytes (Tang, 1999). Defensins has been isolated from various species and from various tissues and secretions. Two novel β-defensin GBD-1 and GBD-2, 64 amino acids long, were identified in the respiratory (GeneBankY17679), and digestive tissues (AJ009877) from a goat, respectively (Zhao et al., 1999). These peptides were identical in 96.8% of their bases and 88.2% of their amino acids. Goat beta defensin-1 (GBD-1) was expressed principally in the tongue and respiratory tract, whereas GBD-2 was expressed throughout the intestine.

Cationic peptides were isolated from goat tongue (Anbu, More and Kumar, 2003), demonstrating their germicidal activity against both Gram-positive and Gram-negative bacteria. Transcripts of GBD-1 and GBD-2 were identified in kidneys, trachea, tongue epithelium, spinal cord, and in mammary gland of non-lactating goats (Bagnicka et al., 2005). GBD-1 was also expressed in the reproductive tract (vaginal, uterus and ovarian tissue) of black goats (Xiaoyan and Wu, 2015). The mRNA sequence of a gene encoding caprine lingual antimicrobial peptide (LAP) was cloned and characterized (Sharma et al., 2010). LAP was isolated from goat tongue epithelium. At nucleotide level goat LAP showed 99.5%, 99.4% similarity when compared with GBD-1, Goat EBD and GBD-2, respectively, whereas at amino acid level Goat LAP showed 98.5%, 87.7% homology with GBD-1 and goat EBD, respectively. Goat LAP is evolutionary closer to GBD-1. LAP is 18 amino acids larger than the GBD-1, while goat enteric β-defensin (EBD) shows similar number of amino acids as in GBD-1.

Caprine enteric β-defensin (EBD) mRNA was cloned and characterized from goat ileum (Kumar et al., 2010). Goat EBD showed 97.4% and 95.4% homology with GBD-2 and GBD-1, respectively at nucleotide level. The amino acids sequence of goat EBD has four and seven substitutions when compared with GBD-2 and GBD-1, respectively. Defensin gene of Assam Hill goat was cloned and characterized (Bharalii et al., 2018) and found to be 64 amino acids long as in case of GBD-1 and EBD. The phylogenetic relationships of different beta defensin from goat has been portrayed in Figure-2 showing the evolutionary relationship of goat β–defensin nucleotides. The concentration of beta defensin-1 was determined in semen pellet and seminal plasma of Indian goat breed namely Barbari, Jamunapari and Jakhrana (Ranjan et al., 2019).

In mammals, β-defensin are mainly expressed and secreted in the epididymis resulting in their detection on the plasma membrane of sperm (Yudin et al., 2005). β-defensin 1 gene has been used as a molecular marker for selection of goats regarding the susceptibility to nematodes and haemoprotozoans infections (Maia et al., 2019). Beta defensin-2 significantly augments the mRNA and protein expression of Toll-like receptors (TLRs) and retinoic acid-inducible gene-I-like receptor (RLR) essential for the detection of viral molecules in mature tissue rat peritoneal mast cells (Agier et al.,

2020). Beta defensin-2 from swine has been investigated for its antiviral efficacy against the pseudorabies virus (PRV), causing Aujeszky's disease (Huang et al., 2020). Concentrations of beta-defensin (GBD-1), cathelicidin (CATH-2, CATH-7), lactoferrin, and S100A7 were determined in goat milk after being fed with colostrum whey using ELISA and it was found that it has a significant effect on their expression and secretion in milk (Isobe et al., 2020). Though defensin plays an important role in host defense against pathogens, recent evidence suggests, that they can also be pathogenic under certain biological conditions by promoting viral and bacterial infections (Xu and Lu, 2020).

Figure 1: Structure of caprine antimicrobial peptides; a. GBD-1 b. Cath-2 (ChBac5) c. Lactoferrin d. Myeloid cathelicidin (Source: Uniprot).

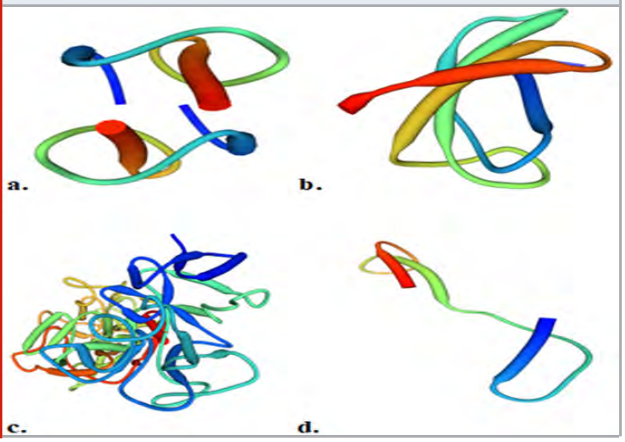
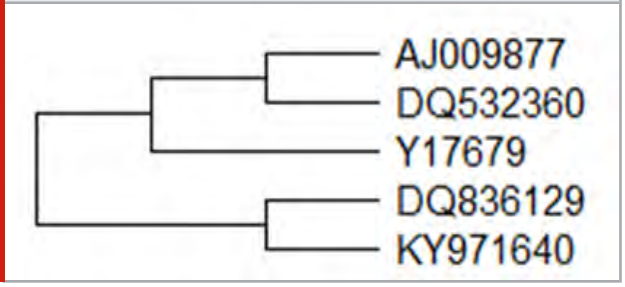
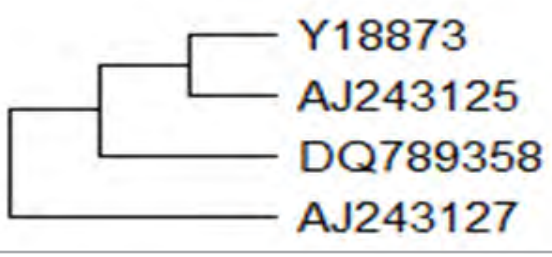


Figure 2: Phylogenetic relationships of different goat β–defensin. A bootstrapped (1000 trials) neighbour-joining phylogenetic tree showing the evolutionary relationship of goat β–defensin nucleotides.



Cathelicidin: Cathelicidins are small, cationic, antimicrobial peptides present in human and other vertebrates. These are proteolytically activated peptides and are part of the innate immune system having a broad spectrum of antimicrobial activity against bacteria, viruses and fungi (Kosciuczuk et al., 2012). It may be used as an adjuvant for vaccine as well as in anticancer therapy by modulating TLR-activation and inflammation (Scheenstra et al., 2020). Cathelicidins are synthesized as prepropeptide, containing a signal peptide, cathelin, and C-terminal mature peptides with antimicrobial properties (Zanetti, Gennaro and Romeo, 1995).

Figure 3: Phylogenetic relationships of different goat Cathelicidin. A bootstrapped (1000 trials) neighbour-joining(Saitou and Nei, 1987) phylogenetic tree showing the evolutionary relationship of goat cathelicidin nucleotides



The mRNA sequence of a gene encoding Bac7.5 and MAP34-A was cloned and characterized from goat (Zhao et al., 1999a). A proline-rich antimicrobial peptide of cathelicidin class was purified from elastase-treated extracts of goat leukocytes namely ChBac5 (Shamova et al., 1999). Ch derives its name from C*apra* h*ircus*. It's a 43 amino acid long peptide with a molecular mass of 5.16kDa. ChBac5 (GeneBank Y18873) was homologous to OaBac5a and bovine Bac5. ChBac5 exhibited potent, broad-spectrum antimicrobial activity against *E. coli, P. aeruginosa, B. subtilis, C. albicans* under low salt concentration. ChBac5 peptides are highly conserved in ruminants and contribute significantly to their innate host defense mechanisms. Another proline-rich peptide (ChBac3.4, 26 amino acid long) was isolated from leukocytes of the goat (Shamova et al., 2009). ChBac3.4 had over 50% sequence identity to the ChBac5 peptides found in the leukocytes of goats, sheep and cattle. ChBac3.4 exhibited broad spectrum antimicrobial activity and also has cytotoxic potential. Mini-ChBac7.5Nα and mini-ChBac7.5Nβ (average molecular masses of 2.89kDa and 2.7kDa) was isolated from neutrophils of the domestic goat (Shamova et al., 2016).

These peptides exhibit significant antimicrobial activity against Gram-negative bacteria. These truncated AMPs may play a crucial role in host defense reactions. The mRNA sequence of a gene encoding myeloid cathelicidin (11.32kDa) was cloned and characterized. This *Capra hircus* cathelicidin (cath) mRNA, partial cds encodes for 99 amino acids and has been isolated from the bone marrow cells of Indian domestic goat (Sharma et al., 2008). Goat myeloid cathelicidin showed 85.9% similarity with ChBac7.5 at nucleotide level. Phylogenetically goat myeloid cathelicidin is closely related to ChBac7.5 than other cathelicidins. The predicted peptide of cathelicidin isolated from bone marrow of Assam hill goat is composed of 137 amino acids (Bharali et al., 2019) and is phylogenetically closer to Yunnan goat cathelicidin (CATH2). The phylogenetic relationships of different goat cathelicidin has been depicted in Figure-3 using neighbour-joining method. ChMAP-28, a cathelicidin antimicrobial peptides from goat (*Capra hircus*) leucocytes having α–helical structure and a molecular mass of 3kDa have been identified, ChMAP-28 has potent anticancer activity (Emelianova et al., 2018).

In another study it was found that the regulation of cathelicidin bovine myeloid antimicrobial peptide (BMAP-28), in the inflammatory response against alpha-herpes viruses is dependent on the stage of virus infection in the bovine nervous system (Burucua et al., 2020). Five cathelicidin mRNAs (Cath-1, 2, 3, 6 & 7) were expressed in deep region of the mammary gland in healthy goats, however, cathelicidin-7 was not expressed in the teat and cathelicidin-2 is expressed in polymorphonuclear cells in the mammary gland and is secreted into milk in goat (Zhang et al., 2014). Goat cathelicidin-2, an antimicrobial peptide, localizes in leukocytes and is present in milk even without lipopolysaccharide stimulation (Srisaikham et al., 2016). Goat cathelicidin-2 has broad-spectrum of activity *in-vitro* against Gram-negative bacteria such as *E. coli* (Shamova et al., 1999). Cathelicidin-1 was also detected in the raw bovine colostrum using LC-MS/MS (Chatterton et al., 2020). The role of cathelicidin as a biomarker in the late lactation period in goats has been demonstrated in relation to mammary gland infection (Puggioni et al., 2020).

Table 1. Expression of antimicrobial peptides in different parts of goat

Peptide or gene name		Tissue(s) Localization	Sources
Defensin	GBD-1	Milk somatic cells,	Zhao et al., 1999
		Tongue, trachea, bronchi, lungs, vaginal, uterus and ovarian tissue, semen	Bagnicka et al., 2005 Xiaoyan et al., 2015 Ranjan et al., 2019
	GBD-2	Kidneys, trachea, tongue epithelium, spinal cord, mammary gland, stomach, jejunum, ileum, large intestine, rectum	Zhao et al., 1999 Bagnicka et al., 2005
	LAP	Tongue epithelium	Sharma et al., 2006 Bharali et al., 2017
	EBD	Ileum	Kumar et al., 2010
	Cationic antimicrobial peptides	Urine	Tomar et al., 2018
Cathelicidin	ChBac5, ChBac3.4	Leukocytes	Shamova et al., 1999 Shamova et al; 2009
	ChBac7.5Nα ChBac7.5Nβ	Neutrophils	Shamova et al; 2016
	Myeloid Cathelicidin	Bone marrow	Sharma et al., 2010
	ChMAP-28	Leucocytes	Emelianova et al., 2018
	Cath-1,2	Polymorphonuclear cells of the mammary gland, Milk	Zhang et al., 2014
	Cath-7	Leucocytes	(Nishikawa et al., 2018)
Lactoferrin	Lactoferricin	Milk	Kimura et al., 2000
S1008		Milk	Purba et al., 2019 Isobe et al., 2020

Lactoferricin / Lactoferrin: Lactoferrin is an important antimicrobial component of milk and it protect the infants from infectious diseases (Reiter, 1978). Lactoferrin also helps in modulation of the inflammatory response, activation of the immune system, and control of myelopoiesis (Brock, 1995). Lactoferrin exert its antimicrobial action by depriving bacteria of the Iron (Arnold, Cole and McGhee, 1977). The presence of antimicrobial domains near the N-terminus of lactoferrin was first reported by (Bellamy et al., 1992) and they named the isolated peptides lactoferricin. Lactoferricin have a broad-spectrum antibacterial property against Gram-positive and Gram-negative bacteria, and fungi. Lactoferrin of goat milk upon pepsin digestion releases a potent antimicrobial peptide called Lactoferricin. This derived peptide is 16 amino acid long and it corresponds

to the sequence of residues 20 and 35 in the N lobe of Korean Native (KN) goat Lactoferrin.

The sequence of the antimicrobial peptide from KN goat lactoferrin showed 75% and 44% similarity with the sequences of the regions between the two cysteine residues of bovine and human lactoferricin, respectively (Kimura et al., 2000). Lactoferrin in goat milk has been confirmed for its role in increasing the activity of natural killer (NK) cells, and increasing the phagocytic activity of phagocytes (Kanwar et al., 2015). Bioactive peptides released during the fermentation of goat milk exhibits antimicrobial activity inhibiting the growth of *E. coli, Salmonella, Micrococcus luteus* and *Proteus mirabilis* (Biadała et al., 2020). It has also been established that the casein phosphopeptides present in goat milk can help in increasing the level of IgA in stool, suggesting a positive effect on mucosal immunity (Kao et al., 2020). Goat milk whey hydrolysate, particularly lactoferrin has been established to possess antifungal activity against at least ten toxigenic fungi from the genus *Penicillium* (Luz et al., 2020).

Hepcidin: Hepcidin (Park et al., 2001) is a cysteine-rich antimicrobial peptide isolated for the first time from human urine and named it hepcidin because of its origin in the liver and its antimicrobial properties. Hepcidin plays a crucial role in regulating iron homeostasis. The role of feeding of fermented goat milk on the expression of hepcidin antimicrobial peptides (HAMP) has been studied and it was found that HAMP mRNA expression was lower in control and anaemic animals fed fermented goat milk with normal iron and also in control and anaemic animals fed fermented goat milk with high Fe content (Moreno-Fernandez et al., 2020). Hepcidins exhibited antifungal activity against *Candida albicans, Aspergillus fumigatus*, and *Aspergillus niger* and antibacterial activity against *Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis*, and group *B. Streptococcus*. *Capra hircus* hepcidin antimicrobial peptide (HAMP), mRNA (GeneBank XM013971234) was predicted by automated computational analysis.

S100A8: S100A8 is a calcium- and zinc-binding protein which plays a prominent role in the regulation of inflammatory processes and immune response. It can induce neutrophil chemotaxis and adhesion. The expression and localization of antimicrobial peptide S100A8 was established in the mammary gland parenchyma, teat, blood leukocytes, and milk somatic cells of goat (Purba et al., 2019). S100A8 protein is one of the important biomarkers in polycystic ovary syndrome (Manibalan et al., 2020).

CONCLUSIONS AND FUTURE PROSPECTS OF ANTIMICROBIAL PEPTIDES

Antibiotic resistance is a big global problem. To thwart this problem, we need to develop new generation of antibiotics and the antimicrobial peptides best fit into this category. These peptides are produced in animals as part of their innate immune response. Antimicrobial peptides have unique ability to be used in conditions like chronic inflammation, wound healing, infectious diseases and multidrug-resistant pathogens. The immunomodulatory activities of these antimicrobial peptides can be exploited in future for the development of vaccines as well as a therapy against cancer and other autoimmune diseases. However, we have to be careful while exploiting these peptides as it may lead to the disturbances in animal's innate defense if the pathogen develops resistance to these antimicrobial peptides.

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Seasonal Variation of Odonate Diversity in Abheda Mahal, Kota, Rajasthan, India

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ABSTRACT

Odonates are fascinating insects and important indicators of water quality and ecological health. They are key organisms of terrestrial and aquatic food webs in form of predators and preys. A study of seasonal variation and diversity of Odonates was conducted at Abheda Mahal of Kota, Rajasthan, India in 2018 -2019. Abheda Mahal is a tourist spot on the outskirts of Kota city with a large pond adjacent to the palace. The site has lush gardens and vegetation. Adult specimens were collected and counted by belt transect method using aerial nets. Specimens were spread, pinned and stored in insect boxes. Identification was done by Zoological Survey of India, Jodhpur, India. The study revealed a total number of 8 species of Odonates belonging to 2 families, out of which 5 species were dragonflies of family Libellulidae and 3 species were damselflies of family Coenagrionidae. *Crocothemis servilia* was most abundant and *Acisoma panorpoides* was least abundant. Abundance of Odonates was highest in monsoon and post monsoon but declined to the least in summer season. The higher Abundance can be attributed to high rainfall and humidity (83%-94%) with temperature range of 24.5^{0}C to 29.5^{0}C, favourable vegetation, perching sites and breeding conditions. The present study provides a baseline data of the site for further taxonomy base research and also for conservational activities. It will also increase interest in research of Odonate diversity.

KEY WORDS: ABHEDA, DIVERSITY, ODONATES, SEASONAL VARIATION, SPECIES ABUNDANCE.

INTRODUCTION

Dragonflies and damselflies are two of the most diversified creatures on the earth. Globally 5,740 species of Odonates are known out of which 474 species in 142 genera and 18 families exist in India (Subramanian, 2014). Odonates are gorgeous insects with aquatic larval forms. Both adult and larval stages are top predators and important elements of the food web (Mishra et al., 2019; Babosova et al., 2019). They serve as an umbrella species in biodiversity conservation. They are also good bioindicators of ecological health (Jacob et al., 2017; Samways et al. ,2016 ; Harisha and Hosetti, 2017; Sahu and Rai ,2019; Ilhamdi et al. 2020).

Kota region is a semi-arid zone of Rajasthan, India. It has many water bodies in and around the rural area which harbour a diversity of Odonates. Abheda Mahal (25^{0}12' N 75^0 47'E) is a tourist park about 8 kms from Kota city. The palace is adjacent to a perennial pond .The palace has lush gardens and vegetation and hence very favourable for variety of Odonates .In the present study a listing of Odonates spotted on the site has been done along with study of abundance and seasonal variation .The latter has been observed in Kota area for the first time .Presently the focus was only on the terrestrial adults .Data on the aquatic larval forms may be investigated in further studies. Odonate diversity has been studied more or less from different areas of India (Das, 2016; Debata et al., 2017; Harisha and Hosetti, 2018; Uniyal et al., 2018;

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Mishra et al., 2017, 2019; Kaur et al., 2020) and in other countries (Koneri et al., 2019; Cannings, 2019; Conniff et al., 2020; Ilhamdi et al., 2020). The present study provides a baseline data of the site for further research and conservation planning. The bioindicator value of the species can be explored. Last but not the least it will also attract attention towards the group of Odonates.

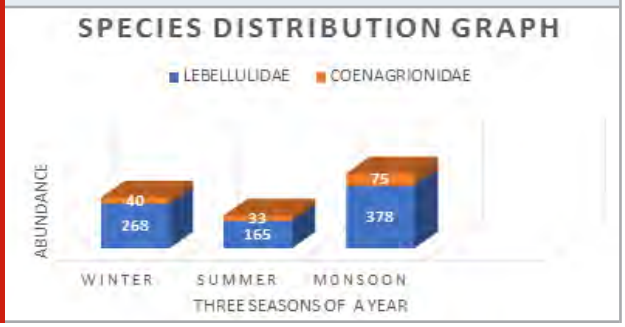
MATERIAL AND METHODS

Abheda Mahal palace is situated just adjacent to a pond and has beautiful gardens. The study was carried out in three seasons during November 2018 to October 2019. Adult Odonate collection was done by belt transect method. All visits and collection were conducted between 9:00 am to 11:00 am. Every month the insects were photographed and also samples were collected from the particular site. Temperature and humidity were also recorded monthly. For the collection of adult Odonates insect net was used. The collected Odonates were stretched and preserved in insect boxes as per standard procedures. The insects were identified by the help of Zoological Survey of India (ZSI), Jodhpur, India.

RESULTS AND DISCUSSION

Observation revealed 8 species of Odonates in the site of Abheda Mahal, Kota, Rajasthan. Out of the identified species 5 were dragonflies (sub-order Anisoptera) of family Libellulidae and 3 species were damselflies (sub-order Zygoptera) of family Coenagrionadae Table 1. The dragonfly *Crocothemis servilia* was the most dominant species which constituted 31.91% of the total abundance followed by *Neurothemis tullia, Branchythemis contaminate*, *Agriocnemis pygmaea, Pseudogrion sp., Rhyothemis variegate, Ceriagrion coromandelianum* and *Acisoma panorpoides* (Figure 1). Libellulidae was the most diverse and abundant family. These observations were more or less similar with the earlier studies (Agrawal, 1957; Harinath et al., 2015; Mandal and Aditya, 2017; Bishnoi and Dang, 2019; Mishra et al., 2019).

Figure 1: Graphical Representation of Seasonal Variation



Abundance of Odonates counted during monsoon were 453 and decreased to 308 in winter and 198 in summer. Higher Abundance can be attributed to high rainfall and humidity (83%-94%) with temperature range of 24.5^0 C to 29.5^{0}C, favourable vegetation, perching sites and breeding conditions during monsoon. This seasonal variation was almost similar to that observed by other authors (Narendra et al., 2016; Thomas et al., 2018; Tuhin ,2018; Nu and Bu, 2019). *Acisoma panorpoides* was rare species spotted only in winters. On the contrary *Pseudogrion sp*. and *Rhyothemis variegate* were most abundant in summer.

Table 1. List of Odonates in Abheda Mahal, Kota, Rajasthan, India

S.NO	SUB ORDER	FAMILY	COMMON NAME	SCIENTIFIC NAME
1	Anisoptera	Libellulidae	Scarlet Skimmer	*Crocothemis Servilia*
2	Anisoptera	Libellulidae	Trumpet Tail	*Acisoma panorpoides*
3	Anisoptera	Libellulidae	Pied Paddy Skimmer	*Neurothemis tullia*
4	Anisoptera	Libellulidae	Ditch Jewel	*Branchythemis contaminata*
5	Anisoptera	Libellulidae	Common Picture *Wing*	*Rhyothemis variegata*
6	Zygoptera	Coenagrionidae	Blue Green Dart	*Pseudogrion sp.*
7	Zygoptera	Coenagrionidae	Pygmy Wisp	*Agriocnemis pygmaea*
8	Zygoptera	Coenagrionidae	Yellow Waxtail	*Ceriagrion coromandelianum*

Table 2. Seasonal Abundance of Odonates in the Abheda Mahal, Kota, Rajasthan, India

S. No.	NAME OF THE SPECIES	SEASONAL ABUNDANCE			TOTAL ANNUAL ABUNDANCE
		WINTER (NOV-FEB)	SUMMER (MAR-JUNE)	MONSOON (JULY-OCT)	
1	*Crocothemis servilia*	93	85	128	306
2	*Acisoma panorpoides*	2	0	0	2
3	*Agriocnemis pygmaea*	36	0	55	91
4	*Neurothemis tullia*	81	11	160	252
5	*Ceriagrion coromandelianum*	3	3	0	6
6	*Branchythemis contaminate*	92	40	79	211
7	*Pseudogrion sp.*	1	30	20	51
8	*Rhyothemis variegate*	0	29	11	40
TOTAL NO OF ODONATES IN ALL FOUR SEASONS		308	198	453	959

CONCLUSION

The present study indicates that Abheda Mahal which is situated in Kota, Rajasthan, India has a rich diversity of Odonate population. The species abundance was found to be highest in monsoon season and lowest in summer season. The most dominant and abundant family was Libellulidae of Anisoptera. Further investigation is necessary for utilizing this group of insects as bio-indicators for managing various water bodies and also used for monitoring environmental changes. Present study is a small contribution of listing and seasonal variation of Odonata of Rajasthan, India.

Conflict of Interests: None

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Chikungunya Virus: New Drug Prospects Emerging from Molecular Docking Studies for Medicinal Biotechnology

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ABSTRACT

The Chikungunya virus (CHIKV) cases were ubiquitously reported in several countries of the North American region, but with time this virus has been spread throughout the world. The Indian subcontinent is not an exception. Till date, the absence of any appropriate drugs or vaccines against the CHIKV makes the research scenario more challenging towards the identification and development of novel lead compounds essential for the same. The Cysteine protease (nsp2) has been identified as a key drug target molecule for combating infections induced by alpha-viruses like the CHIKV. CHIKV nsp2 has an extremely compact structure with RNA-binding surface domains, which make nsp2 more efficient for genome replication during pathogenesis. The present study aims to investigate the novel inhibitors for the nsp2 protein domain using in-silico approach. The Tertiary structure of target protein and various antimicrobial drugs were retrieved from protein data bank and drug bank database respectively. The docking studies are performed and it is observed that Telaprevir is having the highest binding affinity followed by Doxycycline, Sennoside A, Acarbose, and Trobicin. Telaprevir is a widely used antiviral drug for the treatment of chronic Hepatitis c virus. Therefore these drugs can be reprofiled as a potential inhibitor of nsp2.

KEY WORDS: ANTIVIRAL DRUGS, CHIKUNGUNYA VIRUS, MOLECULAR DOCKING, NSP2, DRUG REPROFILING.

INTRODUCTION

Chikungunya (CHIKV) is an epidemic arbovirus that is often used to describe both the virus and the disease. The virus is transmitted mainly to humans through the bite of an infected mosquito of the genus Aedes (Pialoux et al., 1953). The disease generally consists of such a severe infection that cause fever, rashes, and musculoskeletal pain (to walk bent over) is the hallmark of chikungunya that characterizes this dengue-like illness (Staples, Breiman and Powers, 2009; M Dubrulle et al - ‍ 2009; Caglioti et al., 2013; Lo Presti et al., 2014).

There have been several CHIKV outbreaks that have been contributed to describing chikungunya fever in detail and identified maculopapular rash predominantly on the thorax, facial edema a bullous rash with pronounced sloughing, and localized petechial rash. It intensely, affects main extremities, large and small joints eg: ankles, wrists, phalanges (Lo Presti et al., 2014). CHIKV is been carried by an infected female mosquito to the host the mosquito inoculates virus-containing saliva into the bloodstream of a new victim (Lo Presti et al., 2014) (Fig. 1).

CHIKV is an enveloped, spherical body of about 70nm in diameter. The virion genome consists of a Monopartite, linear single-stranded (ss), positive-sense RNA molecule

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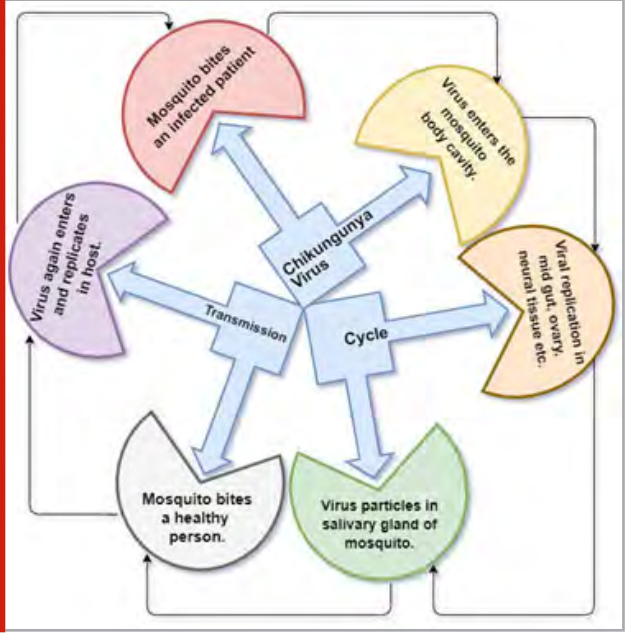
of approximately 11.8 kb long, where the 5' end is capped with a 7-methylguanosine while the 3' end is poly-adenylate. The viral genome contains 2 polyproteins represent four non-structural proteins and five structural proteins (Fig. 2). The replication and propagation of the virus is regulated by nsp2 protein, therefore, it is hypothesized that a compound that inhibits the nsp2 will be a promising and potential drug molecule. In the Era of drug reprofiling efforts can be made to identify a promising inhibitory molecule from the existing antiviral drugs for the treatment of CHIKV the identified potential inhibitors for CHIKV may serve as an inhibitory molecule for other viruses also.which may provide a clear potential path towards the identification of broad-spectrum drugs. (Singh et al., 2011) (Fig. 2).

Figure 1: Transmission cycle of CHIKV

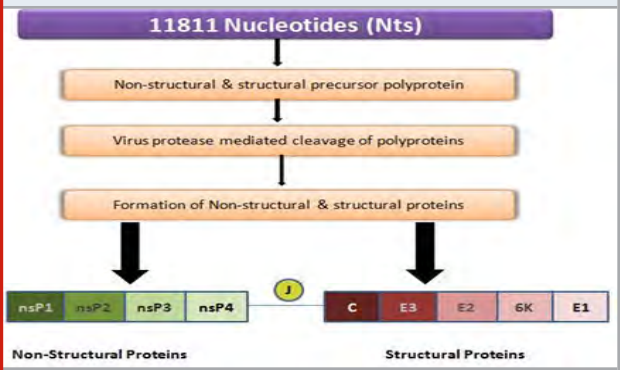


MATERIAL AND METHODS

Retrieval of Target and Lead molecule: The nsp2 crystal structure was retrieved from the protein data bank (PDB) (www.rcsb.org). The retrieval of protein was followed by energy minimization using PYMOL (a user-sponsored molecular visualization system, version 2). The minimization process includes the removal of water molecules, sodium ion, l-peptide linking, and the gaps between amino acids. The lead compound for nsp2 protease was retrieved from PubChem and Drug bank database (Table1). The small molecules were optimized with AVOGADRO: open-source molecular builder and visualization tool(version 1). The optimization process was done with the false parameters that is the force field is off, steps per update is 4, the algorithm is the steepest descent.

Molecular docking studies: Before performing molecular docking studies, we need to identify the binding pockets of the protein molecule. The Automated active site

Figure 2: Schematic description of both structural and nonstructural proteins within the polyprotein CHIKV. CHIKV RNA 11811 bases (top bar, purple color), translates into non-structural and structural precursor polyproteins of 2474 and 1244 residues, respectively, after maturation by protease cleavage, it gives 4 non-structural proteins (left bar, green color) and 5 structural proteins (right bar, red color).



docking and scoring (AADS) is used in this analysis to identify binding pockets. The AADS (http://www.scfbio-iitd.res.in/dock/ActiveSite_new.jsp) utilizes the 3D structure of target molecules and identify top 10 possible binding sites with 100% precision in identifying the real (active) binding sites (Table 4). Once the protein binding pockets are identified, the Small Molecules Library (Table 1) is screened against these sites to identify the hit molecules using the software. For this study, PyRx and AutoDockVina software were used to analyze the ligand-protein binding properties to the protein.

The blind dockings were performed in which the grid boxes' size was adjusted to cover the binding site. Once the docking is complete the resulting PDBQT output file was opened in the PyMOL software for converting all protein conformations into one file analysis on further studies. Afterward, each conformation was examined using Discovery Studio 2.5 software, using information like binding affinities, interaction energies, van der Waals energies, electrostatic energies, hydrogen bonding,pi-pi interactions, pi-cation interactions and close contacting residues were obtained and recorded. The compounds were screened against nsp2 using the PyRx tool to identify the ligands with the best conformers to the target protein.

RESULTS AND DISCUSSION

During Retrieval of the target molecule, the nsP2 with the PDB ID – 3TRK was retrieved and 52 lead compounds were listed (Table1) these lead compounds were screened for potential inhibitory activities against the top 10 binding sites (Table 4) CHIKV's non-structural protein nsp2. The docking studies for the top 10 binding sites of nsp2 (Table3) the docking studies of all 52 lead compounds with 10 binding sites.

Table 1. List of ligands involved in protein-ligand interaction.

S.No.	Drugs	REFERENCE
1.	(R)-Chloroquine	Andersag H et al., 1941
2.	Acarbose	S. P. Clissold et al 1988
3.	Acetaminophen	Kis B et al., 2005
4.	Amikacin Sulfate	Overington JP et al., 2006.
5.	Aspirin	Sneader W ., 2000
6.	Arbidol	Hui Peng et al., 2020
7.	Baicalein (Natural Compound)	Oliveira et al., 2017
8.	Bisdesethylchloroquine	Ajayi FO et al., 1989
9.	Boceprevir	Jennifer J Kiser et al, 2013
10.	Boswellic acid	Arne Henkel et al, 2012
11.	Cefadroxil (Sumacef)	Leonardo Marsili., 1978
12.	Celecoxib	Yi Yu Ke et al., 2020
13.	Chloroquine	Vincent MJ et al., 2005
14.	Cletoquine	Dongre VG et al., 2009
15.	Curcumin	Fatemeh Zahedipour et al, 2020
16.	Desethylchloroquine	Frisk-Holmberg M et al., 1984
17.	Didesethylchloroquine Hydroxyacetamide	Abraham MJ et al., 2015
18.	Dihydrostreptomycin Sulfate	CURCI G ., 1951
19.	Diminazene Aceturate	R. Ghildiyal et al., 2019
20.	Docosanol	Hardman et al 2001
21.	Doxycycline	Dahl EL et al 2006
22.	E-64 (Zinc13493525)	Zheming Wang et al. 2008
23.	Etidronate (Etidronic Acid)	Rogovin et al 1968
24.	Fisetin (Natural Compound)	Liu L et al 2019.
25.	Glucosamine Sulphate	Arvind Chopra et al, 2013
26.	Hesperetin	Samie A et al., 2018
27.	Hydroxychloroquine	Lim HS et al. 2009
28.	Ibandronate Sodium	Epstein S et al. 2005
29.	Ibuprofen	Casper D et al., 2000
30.	Imatinib	Deininger MW et al2003
31.	Iron Sucrose	Hörl WH 2007.
32.	Kanamycin Sulfate	Vetting MW et al. 2002.
33.	Ketotifen	Roy W. Bryant et al. 2011
34.	Leupeptin Hemisulfate	Pérez-Pérez et al 2019
35.	Mitoxantrone Hydrochloride (Novantrone)	Fox EJ 2006.
36.	N-Acetyl (Mono) Desthylchloroquine	E. E. Essien et al1989
37.	Naproxen	Wongrakpanich S et al., 2018
38.	Nelfinavir	Kaldor SW et al 1997
39.	Niacin	Briggs gg, et al.,1998
40.	Officinalis acid	Mohammed Bourhia et al., 2019
41.	Pemetrexed Disodium Hemipentahydrate	Prateek Kumar et al.
42.	Pirodavir	Jef Peeters et al. 2007
43.	Pleconaril	Florea NR et al 2003
44.	Prednisolone	Maryam Daneshpazhooh ., 2020
45.	Quercetagetin (Natural Compound)	Weiyou Wang et al 2016,
46.	Ribavirin	Sidwell RW et al. 2005
47.	Ribostamycin Sulfate	Zhou et al.1992
48.	Sennoside A	Esposito F et al 2016
49.	Sofosbuvir	Asselah T 2013
50.	Spectinomycin Hydrochloride Hydrate (Trobicin)	David R. White 1966
51.	Telaprevir	Kim JJ et al. 2012
52.	Zinc Acetate	Berni Canani R et al 2011

The study suggests out of 52 lead compounds the four compound Telaprevir, Doxycycline, Acarbose, Sennoside A showed significant binding affinity whereas spectinomycin hydrochloride (trobicin), Baicalin, Ibandronate sodium, Quercetagetin, Mitoxantrone hydrochloride, and Fisetin showed promising binding affinity (Table.4 and5.). Telaprevir showed the strongest binding affinity (-12.3kcal/mol), is a member of protease blockers (a group of antiviral medicine). These affinities and energies are due to interaction and bond formation between lead molecules and binding site amino acid of nsp2.

Table 2. Parameters used for molecular docking of top ten ligands with the protein of interest. All grid boxes with a spacing size of 1.000 A˚ have sufficient sizes to cover the entire protein structures during molecular docking.

S.No.	Ligands with Protein	Center-X	Center-Y	Center-Z	Size-X	Size-Y	Size-Z
1.	3trk_Acarbose	12.815566 6274	26.263485 9746	21.59923 82951	82.01098 38983	84.3446 57921	61.15847 77994
2.	3trk_Baicalin	11.6975 98268	23.474 79489	28.50747 38773	67.57002 08963	85.802520 3587	98.212213 4629
3.	3trk_Doxycycline	11.3741 9874	23.059632 7058	21.68560 01381	71.67382 45391	86.78730 81266	53.17848 38552
4.	3trk_Fisetin	28.92522 80574	24.68480 42594	19.22511 98554	115.8926 12756	88.46105 80017	86.8902 924293
5.	3trk_Ibandronate sodium	28.9252 280574	24.6848 042594	19.2251 198554	115.8926 12756	88.4610 580017	86.8902 924293
6.	3trk_Mitoxantrone hydrochloride	28.6614 606151	20.2939 940445	19.3423 23373	104.00 1803984	95.79584 89908	74.5569 102783
7.	3trk_Quercetagetin	12.2522 153681	25.5314 252099	22.5563 495924	70.093 582201	93.8091 943285	81.4775 121373
8.	3trk_Sennoside A	12.2522 153681	25.5314 252099	22.5563 495924	70.093 582201	93.80919 43285	81.47751 21373
9.	3trk_spectinomycinhydrochloride	16.17368 49469	22.7882 300823	17.3275 975767	88.09715 54836	88.323 2612193	73.2683 286745
10	3trk_Telaprevir	12.25221 53681	25.53142 52099	22.5563 495924	70.0935 82201	93.8091 943285	81.47751 21373

Table 3. Cavity details of Nsp2 Protein

S.No.	Cavity Points			V	A	D	R	h	
1.	124.023	55.309	63.320	0.94	0.39	0.42	1.00	0.68	0.6857
2.	100.718	64.861	70.832	0.94	0.56	0.50	0.75	0.63	0.6755
3.	109.046	35.144	85.035	1.00	0.17	0.77	0.50	0.80	0.6465
4.	86.588	66.635	79.871	0.71	0.39	0.46	0.62	1.00	0.6371
5.	92.209	49.198	90.901	0.78	1.00	0.62	0.25	0.41	0.6107
6.	-16.257	-22.551	-4.530	0.98	1.00	1.00	0.83	0.35	0.8326
7.	-23.802	-30.007	2.100	1.00	0.22	0.57	1.00	0.61	0.6804
8.	-7.096	-43.641	-3.465	0.98	0.72	0.53	0.67	0.40	0.6595
9.	-5.740	-45.699	-23.319	0.62	0.72	0.77	0.50	0.48	0.6173
10.	-24.954	-46.789	4.600	0.30	0.67	0.47	1.00	0.53	0.5934

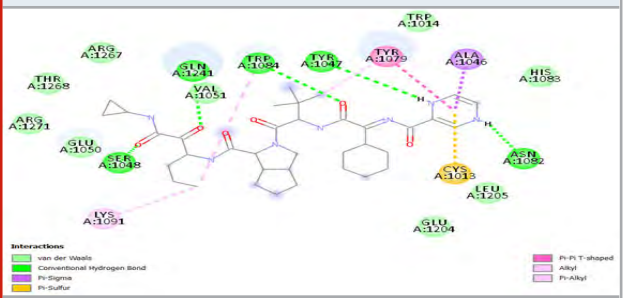
The result shows the amino acid residue found in the binding pocket between Telaprevir and nsP2, are SER1048, GLN1241, TRP1084, TYR1047, ASN1082, TYR1079, ALA1046, CYS1013, LYS1091, GLU1048, VAL1051, ARG1271, THR1268, ARG1267, TRP1014, HIS1083, LEU1205, and GLU1204 a Fig 3. The hydrogen bonds between Telaprevir, Doxycycline, Acarbose, Sennoside A, spectinomycin hydrochloride (trobicin), Baicalin, Ibandronate sodium, Quercetagetin, Mitoxantrone hydrochloride, Fisetin, and 3TRK are also as shown in (Table 4). between Telaprevir and nsP2, are SER1048, GLN1241, TRP1084, TYR1047, ASN1082, TYR1079, ALA1046, CYS1013, LYS1091, GLU1048, VAL1051, ARG1271, THR1268, ARG1267,

TRP1014, HIS1083, LEU1205, and GLU1204 a Fig. 3. The hydrogen bonds between Telaprevir, Doxycycline, Acarbose, Sennoside A, spectinomycin hydrochloride (trobicin), Baicalin, Ibandronate sodium, Quercetagetin, Mitoxantrone hydrochloride, Fisetin, and 3TRK are also as shown in (Table 4).

Table 4. Hydrogen Bonding Between the top hit compounds from the blind docking and CHIKV Nsp2. This table documents the Residues involved in the Discovery Studio 2.5. The binding affinities as ranked by the PyRx 8.0 and Auto Dockvinal 1.5.6 are recorded in the final column of the table.

Ligands with Protein	Hydrogen bonds	AngleDHA(')	Distance(A°)	Binding affinity (kcal/mol)
3trk andTelaprevir	:UNL1:HN - A:ASN1082:O	119.232	2.43416	-12.3
	:UNL1:HN - :UNL1:O	133.404	2.79452	
	:UNL1: HN - A: TYR1047:O	151.652	2.0751	
3trk and doxycycline	A: TYR1047: HN - :UNK0: O	146.817	2.86321	-11.8
	A: TRP1084: HE1 - :UNK0: O	155.005	1.69013	
3trk andAcarbose	A: TYR1047: HN - :UNK0: O	148.924	2.3149	-10.9
	A: SER1048: HG - :UNK0: O	107.507	2.42619	
	A: TRP1084: HE1 - :UNK0: O	147.087	2.46832	
3trk andSennoside A	A: TYR1079: HH - :UNK0: O	152.837	1.82834	-10.9
	A: TRP1084:HE1 - :UNK0: O	135.362	2.37549	
	A: GLN1241: HE22 - :UNK0: O	108.266	2.53045	
3trk and spectinomycin hydrochloride(trobicin)	A: TRP1084: HE1 - :UNK0: O	160.856	2.14139	-8.9
	A: TRP1084: HE1 - :UNK0: O	142.698	2.27101	
	:UNK0: H - A:TYR1079: OH	94.399	2.72027	
3trk and baicalin	A: TRP1084: HE1 - :UNK0: O	150.648	2.03652	-8.1
	A: GLN1241: HE22 - :UNK0: O	99.059	2.87101	
	:UNK0: H - A:TYR1079: OH	138.871	2.70173	
	:UNK0: H - A:ASN1082: OD1	150.896	2.76228	
3trk and Ibandronate sodium	A: TYR1047: HN - :UNK0: O	162.318	2.22615	-8
	A: TRP1084: HE1 - :UNK0: O	132.257	2.66686	
	:UNK0: H - A:TYR1079: OH	102.006	2.77491	
	: UNK0: H - A: TYR1047: O	137.74	2.21746	
	: UNK0: H - A: TYR1047: O	147.153	2.12432	
3trk andQuercetagetin	A: TYR1047: HN - :UNK0: O	149.998	2.84355	-7.9
	A: TYR1047: HN - :UNK0: O	165.015	2.2247	
	A: TRP1084: HE1 - :UNK0: O	135.434	2.27803	
3trk and Mitoxantrone hydrochloride	A: TYR1047: HN - :UNK0: O	157.204	2.30123	-7.8
	A: TRP1084: HE1 - :UNK0: O	173.755	1.82032	
3trk andFisetin	A: SER1048: HG - :UNK0: O	154.341	2.30796	-7.7
	: UNK0: H - A: TYR1047: O	140.152	2.06479	
	:UNK0: H - A:ASP1246: OD2	150.743	2.84791	

Figure 3: 2D diagram of the interaction between telaprevir and nsP2. The diagram shows the ligand- receptor interactions and close amino acid residues found in the binding pocket.

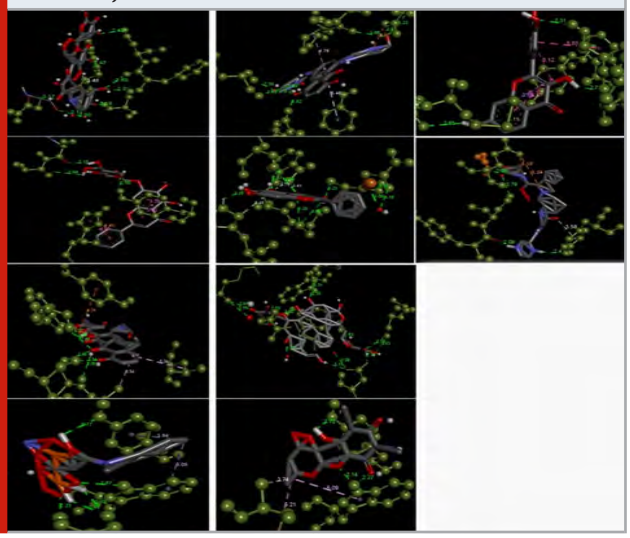


The result of computational studies recommends that Telaprevir, Doxycycline, Acarbose, Sennoside A can be used as nsp2 inhibitors for chikungunya. These lead compounds already exist and were listed in antiviral medicines especially protease blocker so no harm in exploring these drugs for CHIKV inhibition. This significant outcome is for country path in drug reprofiling studies and here we are proposing molecular docking as a tool for exploring new drug prospects from old drugs.

Table 5. Analysis of ligand-receptor interactions

S.No.	Ligand	Binding Affinity	Rmsd/Ub	Rmsd/Lb
1.	3trk_Telaprevir	-12.3	2.456	1.087
2.	3trk_Doxycycline	-11.8	5.49	1.561
3.	3trk_Acarbose	-10.9	5.22	2.49
4.	3trk_Sennoside A	-10.9	8.368	0.016
5.	3trk_Spectinomycin	-8.9	4.485	1.768
	hydrochloride (Trobicin)			
6.	3trk_Baicalin	-8.1	8.143	5.091
7.	3trk_Ibandronate sodium	-8	10.415	9.23
8.	3trk_Quercetagetin	-7.9	31.104	30.446
9.	3trk_Mitoxantrone hydrochloride	-7.8	5.817	0.058
10.	3trk_Fisetin	-7.7	6.404	2.937
11.	3trk_Imatinib	-7.7	21.294	19.022
12.	3trk_Proteinase inhibitor E64	-7.7	12.243	10.903
13.	3trk_N acetyl Desethylchloroquine	-7.6	13.291	11.618
14.	3trk_Nelfinavir	-7.5	28.101	24.91
15.	3trk_Beta-Boswellic acid	-7.5	26.375	23.199
16.	3trk_Etidronic acid	-7.4	2.29	0.784
17.	3trk_Celecoxib	-7.4	5.297	3.179
18.	3trk_Officinalic acid	-7.4	13.228	9.67
19.	3trk_Pleconaril	-7.2	19.127	14.524
20.	3trk_Hesperetin	-7.1	8.237	2.364

Figure 4: The receptor-ligand interactions, and bonds between them with the highest binding affinities of Acarbose, Baicalin. Doxycycline, Fisetin, Ibandronate sodium, Mitoxantrone hydrochloride, Quercetagetin, Sennoside A, spectinomycin hydrochloride, and Telaprevir (Grey, Red, and Blue stick structure) when docked against Nsp2 protein (dark green colored ball and stick structure).



- 3trk_Telaprevir with the binding affinity of -12.3 kcal/mol
- 3trk_Doxycycline with the binding affinity of -11.8 kcal/mol
- 3trk_Acarbose with the binding affinity of -10.9 kcal/mol
- 3trk_Sennoside A with the binding affinity of -10.9 kcal/mol
- 3trk_Spectinomycin hydrochloride with the binding affinity of -8.9 kcal/mol
- 3trk_Baicalin with the binding affinity of -8.1 kcal/mol
- 3trk_Ibandronate sodium with the binding affinity of -8 kcal/mol
- 3trk_Quercetagetin with the binding affinity of -7.9 kcal/mol
- 3trk_Mitoxantrone hydrochloride with the binding affinity of -7.8 kcal/mol
- 3trk_Fisetin with the binding affinity of -7.7 kcal/mol.

CONCLUSION

In our current study, we conclude briefly that Telaprevir, Doxycycline, Acarbose, Sennoside A possesses interactions with CHIKV non-structural protein to (NSP2) which plays a role in the virus replication cycle. These findings enhance our understandings of the possibility of an existing antimicrobial drug molecule to be used for treatment against chikungunya fever. The repurposing of these old drugs to treat chikungunya will become an attractive proposition because it involves the use of no risk compounds with considerably lower development cost and minimal discovery timeline hence further

studies on this target protein and ligands will enhance the development of a novel anti-CHIKV drug.

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Conflict of Interests: We, the authors of the submitted manuscript declare that the work and data present in the manuscript entitled - Chikungunya virus: new drug prospects emerging from molecular docking studies for medicinal biotechnology is genuine research carried out by us. The work finally belongs to the institute. We have not misused the data previously published and have not manipulated the original work.

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Analysing the Response of Non-Coding RNA of Niger, *Guizotia abyssinica* Towards High Temperature and Associated Functional Predictions

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs which regulate gene expression by cleavage or repression of target genes at post-transcriptional level by translational inhibition/ mRNA degradation. Niger (*Guizotia abyssinica*) is an important oilseed crop widely grown in India. Identification and expression of non-coding RNAs during abiotic remains unclear till date. Small RNA library was constructed by high throughput sequencing from control and stress tissues. Target genes of identified miRNAs were predicted using psRNA Target and their GO terms were annotated. The results were validated using RT-qPCR. In this study, we constructed the RNA libraries using next generation sequencing and 125 candidate miRNAs associated to high temperature stress were identified. The qPCR revealed miR395, miR396, miR319 were up-regulated by >15 folds. Most of the targets identified were transcription factors (SPL, MYB, GRF, NAC and GRAS) and oxidative stress. This is to our knowledge the first report for identifying the high temperature stress responsive miRNAs in Niger. Further, characterization and functional annotations of the target genes would provide insights into the regulatory mechanism employed to sustain extreme temperature.

KEY WORDS: ABIOTIC STRESS; GROWTH FACTORS; HIGH THROUGHPUT SEQUENCING; TRANSCRIPTION FACTORS.

INTRODUCTION

Niger (*Guizotia abyssinica*) is an important but neglected edible oil seed crop widely grown in India. Niger is grown in an area of 2.53 lakh hectares with the production of 0.83 lakh tonnes and the productivity of 326 kg/hectare (Dugas and Bartel 2004). The crop of dry areas grown mostly by tribal and desired attention was not accorded on the biotic and abiotic stress conditions. Being a rain fed crop, Niger is exposed many abiotic stresses like drought, high temperature, salt and low nutrients which adversely affect the plant productivity. To date, the reports pertaining to biochemical effects of high temperature or role of miRNAs under high temperature on Niger cultivar is sparse. Thus, it is necessary to elucidate stress tolerance mechanisms by the involvement of miRNAs during high temperature to develop/improve the tolerance cultivar. Small RNAs have emerged as ubiquitous key molecules regulating gene expressionat the post-transcriptional

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level, either by repressing mRNA translation or mediating the degradation of the targeted mRNAs depending on their degree of complementarity (Suryanarayana et al., 2018).

Precursor stem-loop secondary structures are characteristic features of miRNAs and are conserved across species (Carrington and Ambros 2003; Bartel and Sunker 2005). In plants, small RNAs and more specifically, microRNA (miRNA)s (~21 nt), have been functionally associated with development, biotic and abiotic stress (Lelandais-Briere et al., 2009). Regulation of miRNAs by abiotic stresses was initially reported independently by (Jones-Rhoades et al., 2006) and (Sunkar et al., 2004). Subsequently, a number of reports have been published which echoed that miRNAs are themselves regulated by abiotic factors and they in turn, control the levels of target genes involved in governing the stress responses. Two of the most featuring examples are miR398 and miR395, which have been repeatedly shown by independent groups to regulate cellular responses in many different stresses (Li et al., 2010; Khraiwesh et al., 2012).

Bharadwaj et al. (2014) had identified 15 conserved miRNAs in heat stressed Brasicca libraries and validated the expression of miR395 which is indented as another miRNA involved in heat stress response other than miR398 as established in Arabidopsis (Lu et al., 2013) and French bean (Naya et al., 2014). Recently, Kavya and Devraj (2020) have reported the up-regulation of miR166a, miR156, miR6173, miR169e-5p, miR6478 and miR166U under salinity stress. However, till date no reports of miRNA characterization in Niger drawn us towards elucidating the role of in adaptive strategies employed by the oil seed plant to overcome the climatic cues. In this view, we ensued with heat treating the plants at 48 °C for 8 h and profiled their small RNA expression using high throughput sequencing and validating the results with qPCR. We identified 125 conserved miRNAs belonging to 45 families. The cumulative studies of relative quantification using RT-qPCR. Our results emphasize that differential expression would render stress tolerance and has important implications for gene regulation under abiotic stress conditions, (Kavya and Devaraj, 2020).

MATERIAL AND METHODS

Plant material and high temperature stress treatment: Niger seeds were surface sterilized and grown under controlled conditions at 28 °C day/25 °C night with 12 h light/12 h dark photo period. After 6 day of germination, seedlings were exposed to high temperature stress (42 °C for 1 h (induction); 45 °C for 1 h and 48 °C for 6 h). Tissues (shoot) were harvested immediately and stored at -80 °C for further analysis.

Small RNA library Construction and sequencing: Following RNA extraction, small RNA library (control and stress) was prepared according to the True Seq small RNA sample prep Kits protocol (Illumina San Deigo USA). The quality and quantity of total RNA were analyzed using Agilent 2100 bio-analyzer. Ten to thirty nt sRNAs were purified from 15% denaturing polyacrylamide gel and then ligated with the 5' and 3' adapters. After being reverse transcribed by Superscript II reverse transcriptase (Invitrogen, USA), sRNAs were amplified by PCR. High throughput sequencing was performed using Nextseq500 platform (Illumina, USA).

Identification of miRNAs, target predictions and GO analysis: After Illumina sequencing, high quality small RNA reads were extracted from raw reads through filtering the adapter dimers and low-quality tags. Subsequently, unique sequences with 18~25 nucleotides length were mapped with ESTs of Niger precursors in miRBase 21.0 (http://www.mirbase.org/) by BLAST search to identify conserved and novel miRNAs. The potential candidate miRNAs were identified by folding the flanking EST sequence of unique small RNAs using mfold web server (Zuker et al., 2004). Parameters were set based on the criteria for annotation of plant miRNAs by Meyers et al (2008). To identify novel miRNAs, the miRDeep -P program was used to obtain all candidate precursors with hairpin-like structures that were perfectly mapped by sequencing tags. Target predictions were performed using the psRNATarget web server with ESTs of Niger (http://plantgrn.noble.org/ psRNATarget/analysis) using default parameters with a maximum of 3 expectation cut-off. The GO terms of the target genes were annotated according to their biological process, molecular functions, or involvement as cellular components using Blast2GO. The enzyme mapping of the annotated sequences was performed directly using the GO terms and KEGG orthologs.

qRT-PCR analysis of miRNA: RT-qPCR was used to validate the results obtained from the high throughput sequencing of miRNAs. RNA was isolated using Trizol (Invitrogen) as per manufacturer's instruction. 1 µg Total RNA was reverse transcribed using stem-loop primers designed according to Chen 2005 and gene specific primers for target genes using One Step Prime Script miRNA cDNA Synthesis Kit (Takara, Japan). Rt-qPCR was performed using SYBR premix ExTaq (Takara, Japan) and all the primers used were listed in Supplementary file 1. Small nuclear RNA U6 and GAPDH were used as internal controls to normalize the miRNA expression and target genes expression, respectively. Subsequently, the quantification was carried out using (CFX-96, Bio Rad). Three biological replicates were used per sample in addition to technical replicates along with a no template control and no RT-enzyme control. The data were analyzed using 2-ΔΔCT method and reported as means ± standard errors (SE) of three biological replicates. Fold changes were determined by using the ratio of normalized expression of stress against control samples and represented as log 2 values.

RESULTS AND DISCUSSION

Small RNA libraries from stress and control seedlings were screened using Nextseq 500 (Illumina Inc, USA) generated nearly twenty million total raw reads. After

removing low-quality sequences, adapters, and small sequences (< 17 nt long), 18,445,935 and 19,445,620 representing high quality sequences were obtained from stress and control libraries respectively. Further to determine the stress specific miRNAs, the sequences were filtered against the control library. Only reads found in stress library were considered for small RNA identification. An in-house database comprising of non-coding RNAs expect miRNAs from Rfam (12.0) was created and used to filter small RNA fragments corresponding to non-coding RNAs such as tRNAs, rRNA, sncRNAs etc., The small RNA length distribution (16-30 nt) of each library showed that the most abundant and diverse species were those 21-24 nt in length, a typical size range for Dicer-derived products (Fig1) and termed as unique sequences which were further considered for identification of conserved miRNAs. In order to identify the conserved miRNAs, the unique sequences were mapped against mature miRNAs in miRBase (v 21). Following Blastn searches and further sequence analysis, a total of 125 miRNAs belonging to 45 families were identified. miR156, miR399 and miR169(Fig 1) represents the most abundant miRNA family (Fig 2).

Figure 1: Data filtering and Length distribution. A. Pie plot of data filtering B. Sequence read length distribution of mappable high temperature stress responsive small RNAs (sRNAs).

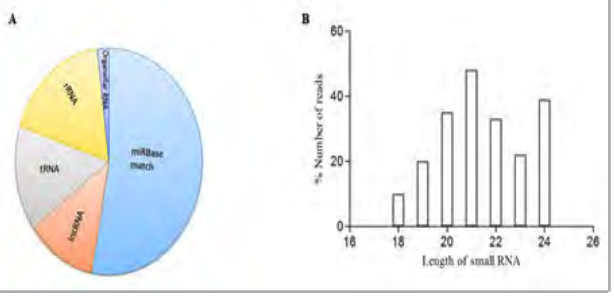
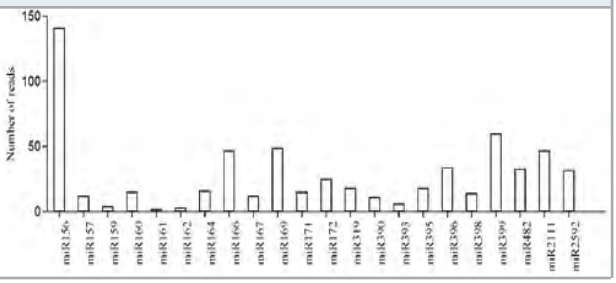


Fig 2: Expression levels of known miRNA families. The expression levels of the miRNA families were normalized by the total number of reads in each of the respective libraries.



All the foresaid conserved miRNA families, possessed miRNA* sequences, identified with their stem-loop structures (Supplementary file of structures) prompts as additional evidence in support of the authenticity of the miRNAs. Since, the precursors and mature miRNAs are highly conserved in plants, it is feasible to define the MFEI values of newly identified conserved miRNAs based on their homologs used as reference sequences. To elucidate the biological functions of high temperature stress associated miRNAs were assessed using the psRNA Target software. A total of 750 potential target genes were identified, based on their perfect or near-perfect complementarity to their target mRNA sequences. Most of the predicted targets were extensively involved in different biological process involving a large number of gene families. Some of these encoded transcription factors, DNA replication proteins, and those involved in cellular metabolism in addition to wide range of stress response associated proteins. Detailed annotations of these results are presented in (Supplementary File 4). miR156 and miR157 targets SPL protein and miR166 targets Homeobox-leucine zipper family protein respectively.

Figure 3: Gene ontology of differentially expressed miRNA targets.

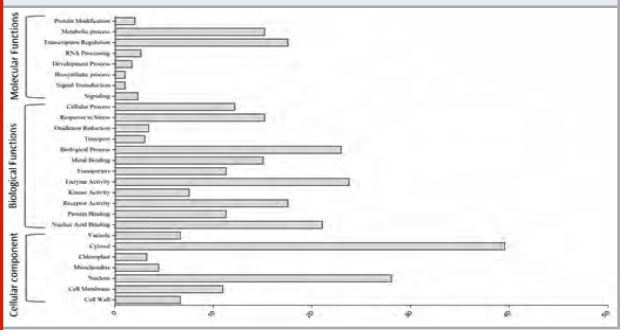
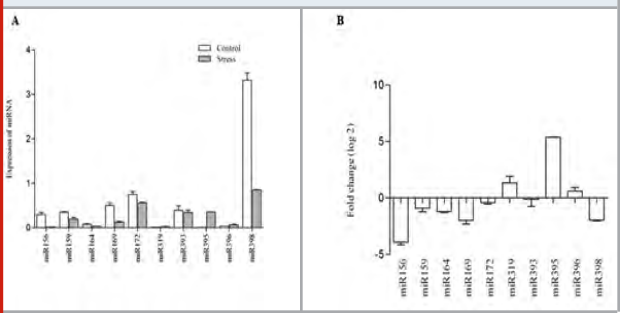


Figure 4: A. Quantification of miRNA abundance via RT-qPCR Differential expression of 10 miRNAs following high temperature stress. B. Fold changes (log2) in known miRNA levels in response to high temperature stress.

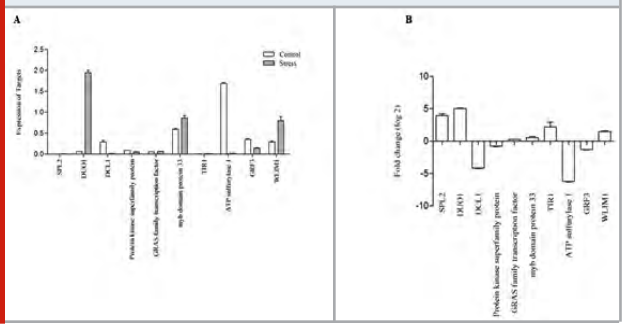


The GO terms of the targets were annotated according to their biological function and highest percentage of genes falls in to cellular components. The enzyme mapping of the annotated sequences was performed using direct GO for the enzyme mapping and the Kyoto Encyclopedia of Genes and Genomes (KEGG) for the definitions of the KEGG orthologs. The miRNA targeted genes belonged to various biological process, cellular components and molecular functions as depicted in (Fig 3). The maximum numbers of target genes were involved in biological process, including both metabolic and cellular process. However, target genes in binding were the most abundant (80%) within the molecular function category. We have also found many genes representing transporters,

receptors and signalling molecules including kinases. The compartmentalization of target genes revealed most of them are membrane proteins and localized in nucleus and cytoplasm.

The putative miRNA targets in Niger were predicted using the psRNATarget program. The target genes (approximately 750 different transcripts) were extensively involved in different biological processes involving a large number of gene families. Some of these genes encoded transcription factors, DNA replication proteins and those that are involved in cellular metabolism in addition to a variety of stress response-associated proteins. miR156, miR166 and miR319 target genes encode Squamosa promoter-binding protein, Homeobox-leucine zipper protein and MYB domain proteins respectively, as previously reported (Ferdous et al. 2015). SPL genes forms one of the most targeted gene and we found 09 SPL genes belonging to Clade-I as major targets from the family. SPLI proteins constitute diverse family of transcription factors which are crucial in plant growth and development. Many studies established the role of SPL proteins in transition of juvenile to adult phase, reproductive transition, trichome development, apical dominance, inflorescence branching, fruit ripening, pollen sac development, and copper homeostasis (Unte et al., 2003; Manning et al., 2006; Wu and Poethig, 2006; Schwarz et al., 2008; Wang et al., 2009; Yamaguchi et al., 2009; Yamasaki et al., 2009; Jiao et al., 2010; Miura et al., 2010; Preston and Hileman, 2010; Yu et al., 2010).

Figure 5: A. Determination of miRNA target gene expression via RT-qPCR analysis of Niger whole Control and heat stressed seedlings B. Fold changes (log2) 10 selected miRNAs and their targets determined by RT-qPCR.



Previous studies established the involvement of miR156, miR157 and miR398 in plant stress response mechanism, specifically in maintaining redox equilibrium under heat stress and copper homeostasis (Lu et al 2012; Preston and Hileman 2013). They demonstrated that, the repression of miR398b and miR398c in Arabidopsis would result in loss of degradation of CSD1 and CSD2, which would support channeling of limited copper to support photosynthetic activity under high temperature induced oxidative stress and copper deficiency. They also showed that the repression of miR156 and miR157 would lead to activation of SPLII genes involved in copper independent photosynthetic pathways. The other major transcription factor targeted by conserved miRNAs is found to be MYB domain proteins.

MYB is one among the vital proteins in plants which involved in development, metabolism, hormone signal transduction, disease resistance and environmental cues (Jagadeeswaran et al., 2009). AtMYB44 was demonstrated to act at front line of salt stress induced cellular re-programming and suggested AtMYB44 prevent stress triggered tissue collapse by rapid removal of ROS (Pashkovskiy and Ryazansky 2013). The most widely targeted class of genes is encoding Toll/Interleukin-1 receptor-nucleotide binding site-leucine-rich repeats (TIR-NBS-LRR). Members of the TIR-NBS-LRR gene family are established as genuine targets for miR2118. Nucleotide Binding Site -Leucine Rich Repeats (NBS-LRR) gene products confers disease resistant in plants and found to harbor secondary small RNAs (tasiRNAs). The miR482 mediated gene cleavage would activate a cascade of defense reactions upon pathogen attack. In cotton, it is demonstrated that, the expression of NBS-LRR was induced to promote the generation of tasiRNAs which mediate small RNA mediated gene silencing, a probable mechanism of plant defence against viral and fungal attack. miR482 and miR2118 were clustered in Medicago trancatula and targets NBS-LRR disease resistant gene (Persak and Pitzschke 2014).

We also observed 10 ESTs coding GRF proteins targeted by miR396. miR396, which targets growth regulating factors (GRFs) are an important class of transcriptional regulators involved in the control of cell proliferation in leaves (Palatnik et al., 2007). RT-qPCR profile showed strong up-regulation of miR396 (1-fold) which appears to play a negative role in cell proliferation due to reduced cell division and it is reasonable to hypothesize that, as in Arabidopsis, miR396 restricts GRF expression, contributing to proliferation arrest in expanding cells (Rodruiguez et al., 2010). Recent studies imply an intriguing regulatory network between miR164, miR396, and miR319 in Arabidopsis: miR319 regulates five members of the TCP transcription factor family (TCP2, 3, 4, 10, 24) that inhibits cell proliferation and directly bind the miR164 promoter region (Khraiwesh et al., 2010; Martin-Trillo and Cubas 2010). Total RNA from the tissues of control and stressed Niger plants were used to validate the miRNAs. The total RNA was converted to cDNA using stem - loop primers. The expression of 10 conserved miRNAs were considered for the study based on their read count/abundancy using qRT-PCR (Martin-Trillo and Cubas 2010).

The expression levels of the Niger miRNAs under high temperature were significantly altered compared with those of the control seedlings. Based on the sequencing data, we selected 10 miRNAs, which were also reported to as stress responsive miRNAs in other species. Of the 10 miRNA we found only 3 of them were found up-regulated. miR395 was found to be highly responsive and was induced by 12 folds when compared with the control seedlings. miR319 and miR396 were up-regulated by 2-folds. Among the down regulated miRNAs, 8-fold

depression was observed with miR156, 4-fold repression was found with miR169 and miR398, and an average of 2-fold repression was observed with other miRNAs (Fig 4). To validate the expression of targets the expression analysis was carried out with selected conserved targets of miRNAs. Since miRNAs were conserved across the kingdom, the genes targeted is also conserved with few exceptions. Since Niger lack complete genome data, we selected conserved targets, for the analysis. The expression profile substantiated the previous observations of negative correlation with their respective miRNAs. ATP sulpharylase was highly repressed by 14 folds, followed by DCL1 and GRF3. DUO1 and SPL7 were induced by 11 and 9 folds (Tian et al., 2014).

However, the target genes exhibited marginal changes in their expression. This may be due to the involvement of transcription regulatory factors other than miRNAs whose expression may not alter due to stress induction (Fig 5). In the present study, RT-qPCR was carried out to study the expression of randomly selected conserved miRNAs representing the most stress responsive miRNA families. All the miRNAs showed sensitivity towards high temperature and our results evidences the miRNA abundance and their expression trends which is consistent with the previous results. We also observed induction of miR395, miR166/167 by 2-fold repression of miR156, miR171 which discern the effects of high temperature on Niger. Many miRNAs were temperature sensitive, for instance, miR160, miR166, miR167 and miR393 were up-regulated in barley and wheat upon heat treatment. Differential expression trends of miR156, miR159, miR396 and miR398 were also observed in Arabidopsis (Jagadeeshwaran et al., 2009) and Broccoli (Tian et al., 2014). miR398 was the most extensively studied miRNA with respect to heat stress. It is demonstrated that the repression of the miR398 under high temperature could render plant tolerant to heat induced oxidative damages (Naya et al., 2014, Lu et al., 2012 and Yu et al., 2012).

CONCLUSIONS

We have identified many non-coding RNAs involved in high temperature stress in Niger were discovered by high-throughput sequencing and annotated their targets. Further, the role of miRNAs target interaction, GO analysis and protein interaction of target gene were studied which showed the identified miRNAs paly an important role in cellular homeostasis in addition to growth and development. However, the detailed mechanism of miRNAs under high temperature stress still requires detailed characterization. However, these finding will contribute for further investigations of miRNAs in Niger under abiotic stress conditions.

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Conflict of interest: The authors declare no conflict of interest.

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In-vitro Plant Production Approach to Increase Heavy Metal Stress Tolerance Capacity of *Polyscias fruticosa*

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ABSTRACT

Pollutants are increasing day by day in the environment. Mitigation of pollutants from the environment is really very difficult task and specially when we are focusing on the soil pollutants, heavy metals are major soil pollutants. Phytoremediation is only the approach by which we can remove the heavy metal from the soil. For that first identification of metal tolerant species is pioneer phase. In this research Heavy Metal stress tolerance capacity of *Polyscias fruticosa* (L.) Harm. was assessed. Here, two different approaches *In-vitro* and *In-vivo* were used for the production of plantlets. In-vitro approach involved tissue culture approach and *In-vivo* direct through media (soil, cocopeat, mosses). Shoot apexes were used for the production of plantlets. After 30 days of seedlings development all the plantlets which are produced through In-vitro and In-vivo approaches and plants were transplanted in the pots and treated with two metals Lead and Cadmium in the form of Pb $(NO_3)2$ and Cd $(NO_3)2$. Different concentrations were selected for Lead 200mg, 400mg, 600mg, 800mg/Kg and for Cadmium 5mg, 10mg, 15mg, and 20mg/Kg. Each pot was filled with 5Kg of soil. The metals were given directly through root zone of plants in solution form. After incubation time of 75 days mature and treated plants were collected and root length, shoot length, number of branches were measured scientifically. On the basis of the results obtained of physiological parameters of the plants we concluded that for both the metals *In-vitro* produced plants has more capacity to tolerate the metal stress as compare to In-vivo produced plants.

KEY WORDS: MICROPROPAGATION, PLANT PRODUCTION, STRESS TOLERANCE CAPACITY, POLYSCIAS FRUTICOSA (L.) HARM, PHYSIOLOGICAL PARAMETERS.

INTRODUCTION

Environmental factors can be of abiotic and biotic nature. Biotic environmental factors, resulting from interactions with other organisms, are, for example, infection or mechanical damage by herbivory or trampling, as well as effects of symbiosis or parasitism. Abiotic environmental factors include temperature, humidity, light intensity, the supply of water and minerals, and heavy metals these are the parameters and resources that determine the growth of a plant. Heavy metals are the major soil pollutants that are emitted from different industries like battery, chemical or steel (Ashwini, Khare and Ganguly 2014). Some plants can survive at high stressful conditions which can be identified and should be grown at high stressful conditions. Some of the stress tolerant plants also has the remediation capacity so the phyto accumulation of the pollutant from the environment can be identified. There are two ways to produce the plants. *In-vitro* production and *In-vivo* production. In *In-vitro* production plants has to be produced under controlled environmental

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conditions like in Green house or in culture room etc. Stress tolerant capacity of all the plants are different. *In-vitro* produced plantlets are healthier and stress tolerant after hardening process (Kozminska et al. 2018).

Polyscias fruticosa (L.) Harm is plant which belonging to Araliaceae family, also known as Ming Aralia. It is dicot shrub native to India. This is shade loving and planted for its foliage purposes. It has compound leaves with seven or more than seven leaflets. Generally, the leaves are deeply lobbed and opposite arrangement is observed. The growth of the plant is seen highest from 19-29°C temperature. Its sensitive plant for any type of stress specially it cannot survive at high temperature. It bears rare flowers and mostly used as an ornamental foliage plant. It is not directly edible by any animals or humans. The leaves have so many important phytochemical constituents and that can be utilized for drug designing. Yang et al. (2009) discovered remediation capacity of Octane through *Polyscias fruticosa* (L.) Harm. Stanley in 2011 described and reviewed indoor phytoremediant plant species and Polyscias was one of the plants that he reviewed. So, here in this research heavy metal stress was provided to the plant *Polyscias fruticosa* (L.) Harm (Yang et al. 2009).

MATERIAL AND METHODS

The shoot apexes (tips) of *Polyscias fruticosa* (L.) Harm were selected for the propagation of plants through In-vitro and *In-vivo* approaches. The Experimental work was completed at Plant Biotechnology Laboratory and Botanical Garden of Gujarat University.

Sources of Explant: Shoot tips were collected with sterilized scalpel from Botanical Garden of Gujarat University. So, shoot tips were used as an explant for the production of plantlets. All the shoot tips were sterilized with the help of 0.1%$HgCl_2$ solution and 70% methanol and rewashed with Grade-1 Distil water (Kanwar, Yu and Zhou 2018).

Aseptic Conditions for Production: Culture room and the laboratory or transfer room were sterilized through Fumigation technique (Potassium iodide and Formaldehyde were used for it with 2:4 ratio). All the glassware and miscellaneous agents were washed with soap solution and rapped with papers and then sterilized through Autoclave (121°C for 20 min). Laminar Air flow hood, weighing scale and all the other small equipment like micropipette were sterilized with 0.1% mercuric chloride solution and 70% methanol.

Preparation of M. S. Media for the production of plantlets: Here for the practical work most widely used media Murashige and Skoog's media (1962) was used. For the preparation first all the Major, Minor, Iron and Vitamin stalk solutions were prepared as per the Table-1. PGRs were not used because in seeds generally we use to avoid PGRs in In-vitro condition and production of plantlets. Here all the chemicals used for the preparation of stalk solution were Hi Media and SRL company (Ijaz et al. 2016). Different stalk solutions were prepared in the amount of 500ml (Major, Minor and Iorn) and 100ml (Vitamin) and then for the preparation of 1 litre M. S. Media 50ml from Major, 50ml from Minor, 50ml from Iron and 10ml from Vitamin stalk were taken and sequentially dissolved and other chemicals which were separately weighed like Myo Inositol, Agar-Agar, Glycine and Sucrose were added for the preparation of media. (Here Grade-1 Purified water was used for the preparation of media with the help of Genie Direct Pure (Rephile) Instrument was used for the preparation of Purified water). After the preparation of media, it was sterilized with the help of autoclave at 121°C temperature for 20 minutes. After Autoclave sterilization the kinetin 0.5mg was added in the media and then under the Laminar Air Flow Hood in all the sterilized culture flasks and Glass jars media was poured about 50ml in each vessel. All the vessels with media were transferred in Culture room where 25±1°C temperature and sterilized conditions were maintained. After 24 hrs media was ready for the Inoculation process (Yang et al. 2009).

Inoculation of Explant: All the sterilized seeds were inoculated separately in the jars or culture flasks under the sterilized conditions of Laminar Air flow hood. Different small equipment was used like forceps and scalpels for the inoculation process. After the inoculation of the seeds in the media all the jars and flasks were again transferred carefully at Culture room where 25±1°C temperature and 16hrs light and 8hrs darkness was maintained. Incubation time was of 40 days.

In-vivo production of Plantlets: By same way sterilized seeds were directly sawed in the media (soil, cocopeat and mosses) in separate pots and regular irrigation process was maintained and up to 40 days the plantlets were produced. The production was carried out at Botanical Garden, Gujarat University. Now same conditions were provided to all the *In-vitro* and *In-vivo* produced plantlets. 40 day's all the plantlets were transferred for the hardening process in the net house of Botanical Garden, Gujarat University where 60% moisture was maintained. Here same media soil, cocopeat were applied for all the In-vitro and *In-vivo* produced platelets. After 40 days in the Net house all the mature plants with 8-12 compound leaves, they were transplanted in different pots separately with 5kg of soil in each pot. In-vitro and In-vivo produced plants were segregated and potted individually in triplicate sets.

Treatment of Heavy Metal to the plants: Lead and Cadmium metals were used for the treatment in the form of Lead nitrate and Cadmium nitrate. For the treatment lead the concentrations were selected 200mg/kg, 400mg/kg, 600mg/kg, 800mg/kg of soil. And for cadmium the concentrations were selected 5mg/kg, 10mg/kg, 15mg/kg, 20mg/kg of soil. One set was kept as control both the series and both the approaches. Lead nitrate and Cadmium nitrate solution series were prepared and the treatment was provided to individual directly through rootzone via digging the soil near by the roots.

Incubation time of the plants: After the treatment to all the *In-vitro* and *In-vivo* plantlets all the plants are placed at Botanical Garden for 75 days incubation period. Regular irrigation was done to all the plantlets.

Table 1. Showing the Composition and Components of M. S. Media (1962) preparation

Stock	Constituents	Quantity		Stock medium
		1 litter (gm)	10 litter(gm)	
A.	Major Stock (gm)			500 ml
	Ammonium Nitrate (NH_4NO_3)	1.65	16.5	
	Potassium Nitrate (KNO_3)	1.9	19	
	Calcium Chloride ($CaCl_2.2H_2O$)	0.44	4.4	
	Magnesium Sulphate ($MgSO_4.7H_2O$)	0.37	3.7	
	Monobasic Potassium (KH_2PO_4)	0.17	1.7	
B.	Minor Stock (mg)	(mg)	(mg)	500 ml
	Potassium Iodide (KI)	0.83	8.3	
	Boric Acid (H_3BO_3)	6.2	62	
	Manganese Sulphate (MnSO4.4H2O)	22.3	223	
	Cobalt Chloride ($CoCl_2.6H_2O$)	0.025	0.25	
	Zinc Sulphate ($ZnSO_4.7H_2O$)	8.6	86	
	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.25	2.5	
	Copper Sulphate ($CuSO_4.5H_2O$)	0.025	0.25	
C.	Iron Stock	(mg)	(mg)	500 ml
	Sodium EDTA			
	(Na2 EDTA.$2H_2O$)	37.3	373	
	Ferric Sulphate ($FeSO_4.7H_2O$)	27.8	278	
D.	Vitamin Stock	(mg)	(mg)	100 ml
	Nicotinic Acid	0.5	5	
	Pyridoxine HCl	0.5	5	
	Thymine HCl	0.1	1	
E.	Myo Inositol	100mg	After the combination of all the required stocks for 1 litter all these weighed chemicals were added in that combination of solution for the preparation of media.	
F.	Glyine	2mg		
G.	Agar-Agar	8mg		
H.	Sucrose	30gm		

Figure 1: Showing *In-vitro* production of aralia

Figure 2: Showing *In-vivo* production of Aralia

Figure 3: Showing Hardening of the plantlets

Figure 4: Showing ready plants for transplantation

Figure 5: Showing Treatment to the plants in pots

Figure 6: Showing Plants after metal treatment

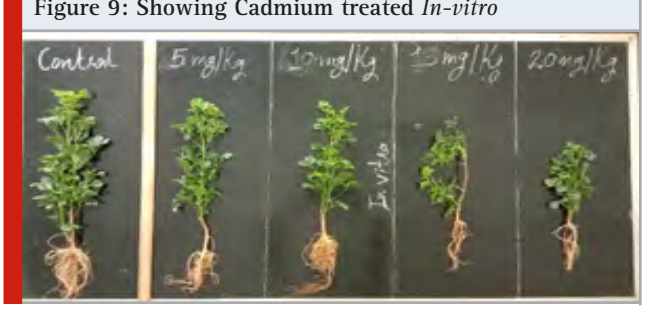
RESULTS AND DISCUSSION

After 75 days the plants were taken out. Different parameters like root length, shoot length and total no. of branches were measured and counted. As the table data and graphical representation shows that as the metal concentration increases the root length, shoot length and no. of branches were decreased. For *In-vitro* plants lead 200mg concentration in the soil plants growth parameters showed 20.4cm root length, 36.8cm shoot length and 4 number of branches but as the concentration increases 800mg concentration in the soil showed that decreased plant growth included 13.9cm root length, 29.8cm shoot length and 2 number of branches. For In-vivo plants lead 200mg concentration in the soil showed 15.9cm root length, 33.9cm shoot length, 3 number of branches and 800mg concentration of lead showed decreased physiological parameters of the plant included 9.8 root length, 20.8 shoot length and 1 branch.

Figure 7: Showing lead treated *In-vitro* plants

Figure 8: Showing Lead treated *In-vivo* plants

Figure 9: Showing Cadmium treated *In-vitro*



For Cadmium *In-vitro* produced plants at 5mg concentration in the soil showed 15.3cm root length, 40.2cm shoot length, 3 number of branches and highest concentration 20mg in the soil showed that decreased physiological data included 12.3cm root length, 32.0 cm shoot length and 2 number of branches. For *In-vivo* plants cadmium 5mg concentration in the soil showed at 15.9cm root length, 32.9cm shoot length, 3 number of branches and highest concentration 20mg in the soil showed 9.1cm root length, 28.4cm shoot length and 2 number of branches. Even the cadmium effect on the plant growth was more than the lead because at lower concentration also it showed the effect on the growth parameters of the *Polyscias fruticosa* (L.) Harm (Kays 2011; Waoo, Khare and Ganguly 2014).

Figure 10: Showing Cadmium treated In-vivo plants

Table 1. Showing Effect of Lead on the *In-vitro* produced *Polyscias fruticosa* (L.) Harm

Treatment Series	Root length (cm)	Shoot length (cm)	Total No. of Branches
Control	22.2	40.6	5
200mg/kg	20.4	36.8	4
400mg/kg	16.8	35.4	4
600mg/kg	15.1	31.9	4
800mg/kg	13.9	29.8	2

Table 2. Showing Effect of Lead on *In-vivo* produced *Polyscias fruticosa* (L.) Harm

Treatment Series	Root length (cm)	Shoot length (cm)	Total No. of Branches
Control	17.4	38.7	4
200mg/kg	15.9	33.9	3
400mg/kg	13.2	30.1	2
600mg/kg	11.6	24.3	1
800mg/kg	9.8	20.8	1

In the comparison of *In-vitro* and *In-vivo* produced plants In-vitro produced plants has more capacity to tolerate metal stress because the growth of these plants was observed higher than the In-vivo produced plants. As the table shows that as compare to control the metal stressed plant growth rate of the plants (root length, shoot length, no. of branches) were lower for both the metals lead and cadmium (Mojiri et al. 2013). Thach et al. (2016) worked on the propagation of the plant as a medical plant because of the volatile compounds found in the leaves of *Polyscias fruticosa* (L) Harm. Boye et al (2018) discovered the effect of the extract of the plant in male rat.

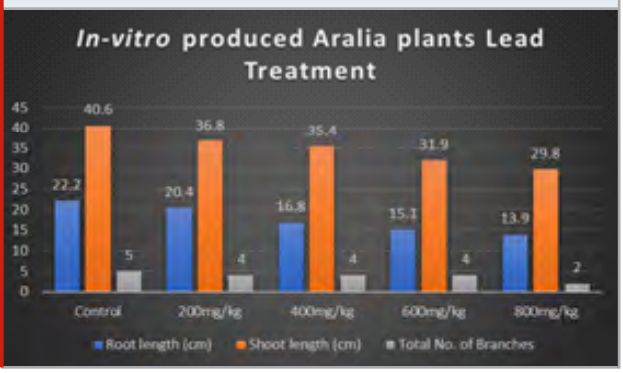
Table 3. Showing Effect of Cadmium on *In-vitro* produced *Polyscias fruticosa* (L.) Harm

Treatment Series	Root length (cm)	Shoot length (cm)	Total No. of Branches
Control	18.9	42.6	6
5mg/kg	15.3	40.2	3
10mg/kg	14.9	33.5	3
15mg/kg	13	32	2
20mg/kg	12.3	29	2

Table 4. Showing Effect of Cadmium on *In-vivo* produced *Polyscias fruticosa* (L.) Harm

Treatment Series	Root length (cm)	Shoot length (cm)	Total No. of Branches
Control	18.8	36.9	4
5mg/kg	15.9	32.9	3
10mg/kg	13.7	30.8	3
15mg/kg	11.8	29	2
20mg/kg	9.1	28.4	2

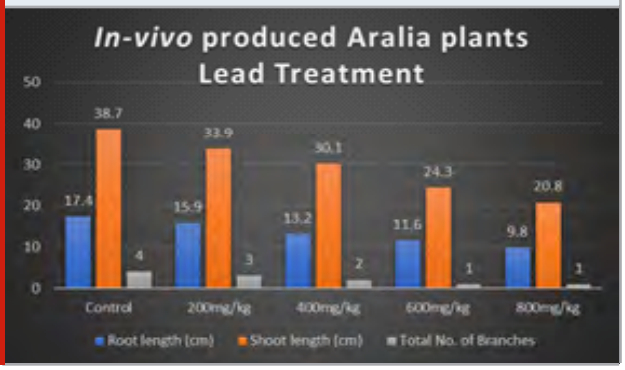
Graph 1: Showing Effect of Lead on the *In-vitro* produced *Polyscias fruticosa* (L.) Harm



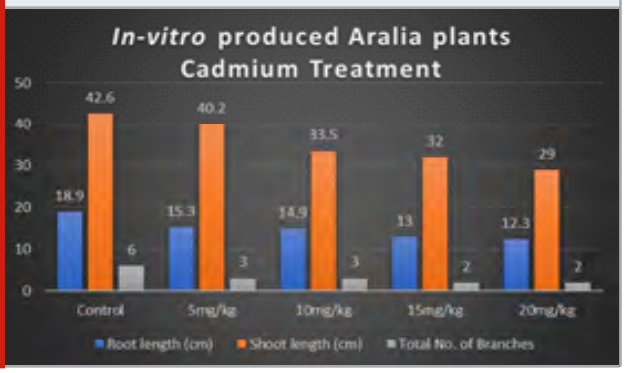
Koffur et al. (2014) discovered the anti-inflammatory effect of the plant. Salva S. Sakar et al. (2014) worked on *In-vitro* production of *Polyscias fruticosa* (L) Harm. In this research work heavy metal stress was provided to the

different method (*In-vitro* and *In-vivo*) produced plants. So many researchers worked on the phytochemicals and different activities of Polyscias fruticosa (L.) Harm there was no any record or the review of articles which showed heavy metal or stress tolerance activity or its effects on the growth parameters of the plant. In future the proteins or the phytochemicals can be identified which are responsible to increase the metal stress activity of the plants. For this study in future phytoremediation study can be assessed of the plants and with the help of In-silico analysis the binding capacity of metal and plant proteins can be analysed and protein molecules can be identified where the metal binds strongly (Hussain et al. 2018).

Graph 2: Showing Effect of Lead on the *In-vivo* produced *Polyscias fruticosa* (L.) Harm



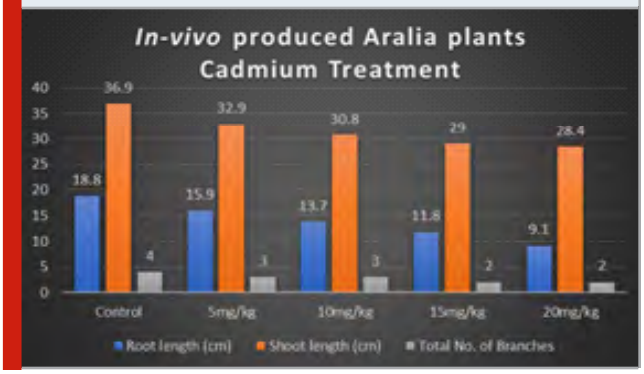
Graph 3: Effect of Cadmium on the *In-vitro* produced *Polyscias fruticosa* (L.) Harm



CONCLUSION

Polyscias fruticosa (L.) Harm is highly metal stress tolerant plant. The plant can survive at high lead and cadmium stress. *In-vitro* produced plants has more capacity to tolerate metal stress compare to In-vivo produced plants. Cadmium effect on the plants was higher as compare to lead metal stress. The research is also coming up with new application of plant tissue culture too increase metal stress tolerance capacity. After the treatment the plant material can be used for the production of Biochar which can be used in different industries like tier industries, varnish industries. After the identification of proteins which provided stress tolerant activity to the plants, the

Graph 4: Effect of Cadmium on the *In-vivo* produced *Polyscias fruticosa* (L.) Harm



genes which are responsible for the production of that proteins can be identified and with the help of genetically modified technology it can be inserted in another plants and stress tolerant plants can be produced. So, it can be applicable to molecular genetics level and to improve the crop stress tolerant capacity.

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Food Management During Hajj Using Lean Methodology to Fulfill the Pilgrims' and Umrah Performers' Food Needs, Rationalize Consumption and Preserve the Environment

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ABSTRACT

Hajj is an obligatory religious duty. It is one of the greatest and closest rituals to the Muslims' hearts. Saudi Arabia's government is exerting continuous efforts to serve the Pilgrims and Umrah Performers, provide them with their needs, especially quantities and qualities of food for maintaining good health and develop scientific and practical solutions for any emerging problems to achieves their security, safety, and well-being. Besides, enable them to perform their rituals with ease and tranquility. Therefore, the question of this research is to ensure the provision of nutritional needs appropriate with the different health conditions of the Pilgrims, where improper nutrition affects their health, physical fitness and hinders the performance of the rituals. Also, improper food rationalization leads to a massive surplus of the remaining food, thus inducing environmental pollution, accumulation of microorganisms, and the reproduction of insects that transmit toxins and diseases. This research aims to apply food management during Hajj using the Lean Methodology to ensure fulfilling Pilgrims' needs according to their health status, rationalizing the consumption, and preserving the holy rituals' environment against pollution. The research follows the descriptive and analytical approach; two questionnaires were used to obtain the results of the study. There was 20–40% waste food, their cost ranging between 1.3-3 million SR, which means considerable amounts in total and edible leftovers are not used optimally. Results of Pilgrims' response to the second part of the questionnaire to obtain proper nutrition and reduce waste of food indicated pilgrims' approval of most proposals had ranged between 75 - 100%.There were huge amounts in total and edible leftovers, which are not used optimally. Therefore, applying the Lean Methodology was a suggested step for Pilgrims and Umrah performers to achieve their nutritional needs and reduce food waste.

KEY WORDS: FOOD MANAGEMENT- HAJJ - LEAN METHODOLOGY - NUTRITION NEEDS - FOOD WASTE - ENVIRONMENTAL PROTECTION.

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INTRODUCTION

The large crowds of Pilgrims and Umrah Performers are in dire need of healthy, balanced, nutritious food that provides them with their nutritional needs. The Hajj or Umrah spiritual journey requires exerting physical effort comfortably, without headache, tension, anger, and exhaustion. Hence the key role of a balanced healthy diet, especially with changing sleep and mealtimes. Proper nutrition for Pilgrims and Umrah Performers is one of the essential factors for maintaining health and body strength, though it might also be one of the causes of its weakness and illness. Therefore, it is necessary to raise the level of food management and rationalize its consumption for pilgrims for their activity and prepare them to perform the rituals easily and smoothly without suffering from any weakness.

As low vitality or facing health problems resulting from malnutrition may not suit their health and physical condition, in addition to wasting quantities of food instead of utilizing them. Hajj travel agencies tend to provide very large quantities of food that exceeds the actual food needs of the Pilgrims and Umrah Performers, seeking to please them without consideration to the waste of food and the resulting health problems for the Pilgrims that lead to a decrease their ability to perform the rituals appropriately (Qanta et al., 2006; Bond et al., 2013). Consequently, it is necessary to raise the level of food management and rationalize its consumption for Pilgrims to renew their activity and prepare them to perform the rituals smoothly and easily without suffering any weakness, lacking vitality or facing health problems, nor wasting any quantities of food (Vankatesh and Memish, 2017 UNEP, 2019).

Food management begins with defining the goal towards proper nutrition for Pilgrims, which helps them to carry out the various rituals of Hajj with the necessary vigor, prevent several health problems that arise from providing them with inappropriate nutrition. Planning for the meals come in two parts. First: planning for proper nutrition, including determining the health conditions that must be met in each meal, in terms of specifying its quantity and components, to suit the different groups of Pilgrims (healthy, patients with chronic diseases such as hypertension, diabetes, kidneys, elderly persons, children, and pregnant women) and meet their bodies need of energy and nutrients, with a focus on micronutrients to increase the body's' immunity and its ability to resist infection.

In addition to determining the places from which the components of the meals are purchased, selecting the most appropriate preparation and cooking methods that maintain the highest nutritional value of the meals provided. The methods of packaging, preserving, and storing that ensure handing over healthy meals, considering the compatibility if such meals with the dietary habits of the Pilgrims. Second: planning for food spending so that proper nutrition would be available at the lowest possible expenses; the more spending on food items, operation costs increase and Pilgrims bear more expenses that could be avoided by adopting the methods and techniques of rationalizing spending on food during Hajj (Yamin, 2007 UNEP 2019). Food losses and waste is an emerging issue with huge environmental, social, and economic implications (WRAP, 2008; WRAP, 2012; FAO, 2013; HLPE, 2014; WRAP, 2020).

Meals management includes the method of distribution, manners of directing and guiding the Pilgrims to choose the types and quantities of food that are appropriate to their needs and health conditions optimally, to keep the energy required during the performance of Hajj and to avoid the occurrence of digestive disorders as a result of poor food selection or excessive eating. In addition to guiding them to how to preserve the leftovers and eat later or redistribute them to the needy, as well as to the healthy ways to get rid of the waste after consuming the meal (Shaikh-Omar et al., 2013).

Implementation and control in terms of adherence to the sound rules required for feeding Pilgrims by Pilgrims supervisors, tour guides, and the persons in charge of serving them in the relevant travel agency, in addition to allocating places for eating, away from the areas where Pilgrims sleep, adhering to the best methods of choosing, buying, preparing, cooking, canning, packaging, preserving and storing food, according to health conditions. Also, compliance with healthy practices in re-packaging, preserving, and transporting the edible leftovers for distribution to the needy and the healthy ways to get rid of food waste and non-edible remnants. Finally is the role of evaluation of each of the previous stages and re-examining them to identify the elements of success and failure in meals management, to assess the provision of the next meal by adopting and emphasizing the methods of success and avoiding the practices that led to obstacles in food management, replacing them with ways and means support better management (Jaralla et al., 1993; Yousef et al., 1995; Al-Mazrou, 2004).

Lean Methodology is a management philosophy that appeared in Toyota after the Second World War at the hands of the scientist Taiichi Ohno in Japan, because of the urgent need to cover the deficit in the capital. That methodology was based on reducing waste and making the possible use of available resources (Womack et al., 1990). The use of Lean Methodology started since 1990 on a large scale in various Japanese companies for the disposal of waste, surplus, and non-value-added activities in the operations, as well as to change the corporate culture towards continuous improvement and increased customer satisfaction (Pepper and Spedding, 2010). Lean Methodology can help companies to dispose several types of waste, the most famous of which are the seven types of waste: "movement", "unnecessary transportation", "overproduction", "over-operation", "time", "rework" and "inventory" (Ohno, 1988). Waste is disposed of by using a set of simple non-statistical Methodology and techniques, such as "Cause and Effect Map", "Value Stream Mapping", "5S", "Kaizen",

"Brainstorming", "SIPOC", "Checklist", and "5 Whys" (Antony et al., 2003).

Many experiences indicated the importance of applying Lean Methodology in both industrial and service companies, due to the positive results achieved in disposing of or reducing waste while best utilizing the available resources to increase production. The study by Bowen and Youngdahl (1998) used lean application in some restaurants in the USA to increase food preparation efficiency, increase customer satisfaction, and speed up the food preparation process. By using value chain analysis and JIT. It is thus reduced kitchen space by dispensing with unnecessary appliances, rapidly preparing food, and serving it at an appropriate temperature and high quality, harmony of employees in the performance of work, and increasing customer satisfaction. The obstacles contain great diversity in customer needs.

Engelund et al. (2009) used Lean application to increase the efficiency of food production, reduce food production steps, thus increasing production speed while maintaining quality, reduce the number of employees in the kitchen, and reduce waste of raw materials used in preparing food, thus reducing expenditures by using value stream mapping, 5S, and Kaizen. They are thus reduced the number of employees in the kitchen from 71 to 54, increasing the efficiency of food production steps, ensuring an enjoyable work environment for employees, and reducing waste in food products to 5% by switching to production on demand. The obstacles contain employees with different nationalities and languages, leading to difficulty in communication. Mohammad (2017) used Lean application to improve the operations related to the savings in the hotel, outperform competitors, and reducing operating expenses related to food production.

By using value stream mapping, which leads to increase customer satisfaction by understanding their needs, reduce the costs of food management operations by excluding unnecessary steps, improve the quality of food and beverages served by setting quality standards based on customer needs, and outperform competitors by providing high quality food at affordable prices. The obstacles contain convincing some administration employees of the importance of using Lean Methodology, failure of some employees to implement the proposed improvement, and difficulty finding the right time for all participating employees to start improving together.

The mentioned researches show the feasibility of using Lean Methodology for the service product to help reducing waste of foodstuff used in food, improve its quality, as well as lowering the Methodology, equipment, and devices required for speeding up the preparation process, reducing unnecessary steps to decrease the time and effort of the workers, in addition to achieving other benefits for the service consumer, including increasing customer satisfaction in terms of food quality, price, temperature, cooking methods, food preferences, by using some simple Methodology. On the other hand, the experiments and studies that dealt with the application of Lean Methodology to reduce the waste of food quantities provided in places with large human gatherings, such as residential homes, hospitals, student housing, and school that provide meals.

There are several Methodology adopted in applying Lean Methodology, the most suitable tool to achieve the goal of this research in meeting the needs of Pilgrims and reducing food consumption is the use of the Deming Cycle (Plan-Do-check-Act cycle, PDCA) (Pratik and Vivek, 2017) which designed by the scientist Deming in 1951 to be one of the most essential Methodology used to achieve continuous improvement in production, as well as maintaining the sustainability of continuous improvement and learning from mistakes and previous experiences. The cycle consists of four main steps: Plan: the first step is planning, i.e., presenting the necessary strategies to improve quality after identifying the problem and collecting and analyzing the essential data; Do: implementing the plan and applying the change in a limited scale; Study: measuring and evaluating results and determining whether the improvement efforts were successful or not; and Act: if the results are successful, then adopt the improvement plan and apply it to other areas in the organization. If the results are unsuccessful, then amend the improvement plan (Johnson, 2002). Therefore, this research assumes that there was a failure to meet the nutritional needs of the food provided to Pilgrims during Hajj, with foodstuff improper to their health conditions.

METHODOLOGY

The descriptive and analytical methods were used to represent the obtained data in the present study.

Research Methodology: Two questionnaires were applied in this research The first questionnaire: Meeting the nutritional needs with reducing food waste during Hajj and Umrah, was addressed to the organizers and guides of campaigns responsible for preparing for Hajj and Umrah tours. The number of (25) of the operators and tour guides, whose experience ranged from 4-35 years, with Pilgrims in their campaigns ranging from 400 – 2000 persons. The second questionnaire: Meeting nutritional needs while reducing food waste during Hajj and Umrah, was addressed on Pilgrims from both male and female, with age more than 20 years who performed Hajj from no more than 4 years. The questionnaire including questions about gender, age group, time from last Hajj, Pilgrims category, and their health status (Appendix 1). After obtaining the data, a proposal presented to apply Lean Methodology with the Deming Cycle to meet the nutritional needs of Pilgrims and reduce waste was induced (Appendix 2). Statistical analysis : Data are presented as frequencies and percent using SPSS.

RESULTS AND DISCUSSION

Appling the study Methodology on the Pilgrims, Hajj agencies, and their guides revealed that the waste food ranged from 20% to 40% of the total quantities of food, with a cost ranging between (1,300,000 – 3,000,000 SR), which means huge amounts in entire and edible leftovers are not used optimally. Food losses and waste is an emerging issue with huge environmental, social, and economic implications (WRAP, 2008; WRAP, 2012; FAO, 2013; HLPE, 2014; WRAP, 2020). It undermines the basis of food security (FAO, 2011; FAO, 2017; Smil, 2004; Kummu et al., 2012). The reduction of food waste is also considered crucial to decrease the food-related environmental footprints (FAO, 2011; Kummu et al., 2012; HLPE, 2014; UNEP, 2019). Indeed, food waste amounts to a significant depletion of resources (including both natural resources such land and water and other economic resources such as energy and capital) at global and local levels (FAO, 2011; Kummu et al., 2012; Bellù, 2016]. Food waste also represents a considerable loss of money for all food supply chain actors, including producers and consumers (Rutten, 2013; Lipinski et al., 2016).

Table 1. Demographic characteristics of the study participants (n =250)

General characteristics	Frequencies (n=250)	Percent %
Gender Male	52	20.6
Female	198	79.4
Age groups		
20-29	28	11.1
30-39	72	28.6
40-49	87	34.9
50 Y and above	63	25.4
Time from last Hajj Less		
than 1 Y	95	38.1
One Y	99	39.7
Two Y	24	9.8
Three Y	28	11.14
Four Y	4	1.26
Pilgrims/category		
Category (A)	15	6.1
Category (B)	18	7.2
Category (C)	60	24
Category (D)	28	11.1
Category (E)	-	-
Category (F)	129	51.6
Health status No diseases		
Have diseases	140	56
	110	44

Data present as frequencies and percent (total number=250).

In the present study, the results revealed that only 24% of Hajj Agencies were dependent on a resident nutrition supervisor in the Agency, despite the importance of his presence. There is no one to guide the Pilgrims regarding the food suitable for them, in terms of quantity and varieties, according to their needs and health conditions. Only 12 % of the Hajj Agencies served special meals for those with chronic diseases, while for sensitive groups, like elderly and children, only 8% specific meals are taken into consideration. In this study, some of the cooking methods used and the types of food provided to Pilgrims cause digestive disturbances in 55.38% of them. Food waste and leftovers are spreading in the environment surrounding the camps at a rate of (70.8%) and in the areas of the holy sites, which leads to visual and environmental pollution, the spread of unpleasant odors, and hindering Pilgrims smooth movement during the performance of the rituals.

There is a controversy about the incidence of various diseases and health problems during the occasion of Hajj. However, gastrointestinal complaints (GID) were found to be one of the most typical disorders during Hajj (Khamis, 2008). The occurrence of foodborne diseases comprising food poisoning in Hajj in Saudi Arabia is commonly recognized just after or a certain period after taking the meal (Jaralla et al., 1993; Malik et al., 1993; Kurdi, 1995; Al-Awaidy and Fontaine, 1996; Gaulin et al., 2002; Al-Mazrou, 2004; Heymann, 2004). The outbreak of food poisoning directly or indirectly relates to food handlers (Angelillo et al., 2000; Maguire et al., 2000). Diarrheal diseases in pilgrims during Hajj performance might occur due to inappropriate standards of food hygiene, low storage of many foods. However, it was the third most common cause of hospitalization (Al-Ghamdi et al., 2003).

Results of Pilgrims' response to the second part of the questionnaire, "Pilgrims proposals to obtain proper nutrition and reduce waste of food" indicated that 20.6 % of the participants were males, and 79.4 % were female. The majority of the participants, 34.9 % were in the age group 40-49 Y, followed by 28.6 % in the age of 30-39 Y, then 25.4 % were 50 Y and above, while 11.1 % were in the age group 20-29 Y. Concerning the time from last Hajj the majority of Pilgrims, 77.8 %, were 1 Y or less, while 11.14 % were three Y, 9.8% were two Y, and only 1.26 % were four years from last Hajj. Regarding the disruption of Pilgrims per category the majority of the participants 51.6 % were in category (F), 24% were in category (C), 11.1 % were in category (D), 7.2 % were in category (B), while only 6.1 % were in category (A). Concerning health status 56 % of the participants were healthy and 44 % of the participants having diseases as diabetes, hypertension, liver diseases, renal diseases, and gastrointestinal diseases Table (1).

The results revealed that 75 - 100% of Pilgrims' approval of proposals items including:

- The presence of a supervisor or nutritionist in each Hajj agency to provide them with nutritional counseling.
- The necessity of having training courses in "optimal nutrition for Pilgrims " for supervisors in Hajj agency

before the season of Hajj in sufficient time.

- Preparing special meals with considered the quantity of food for sensitive groups of Pilgrims as elderly, children, or they have health condition affected on their nutritional needs. These packaged and standardized meals can sell with low cost in fast food stores in the holy sites.
- Using healthy cooking methods in preparing foods for Pilgrims, such as steaming and grilling, avoiding fried foods and thick sauce food, as much as possible.
- Using large screens and signs spreading in the areas of the holy sites to advice regarding the need to rationalize food and reduce food waste.
- Recycling of food inedible leftovers in making fertilizers and animal feed.
- Avoiding repeating the food types that do not accept by Pilgrims to reduce the food waste.
- Focusing on fresh vegetables and fruits in the meal instead of rice and the various kinds of starches.

Table 2. The proposed steps to implement Deming Cycle and Lean Methodology to provide the Pilgrims' nutritional needs and reduce food waste

Stage	Aim	Example	Lean Methodology to be Used
Plan	1. Defining the problem 2. Analyzing the problem based on the information gathered	First Problem: Failure to meet food needs of the Hujaj, especially those with chronic diseases. Second Problem: Huge waste of food provided by travel agencies to Hujaj. 2. The results of the questionnaire indicate that 49% of the sample are dissatisfied with the quality of the food provided to them, while 80% of the sample agreed that there is food waste in most agencies. At this stage, a strategy must be proposed to collect real and correct information on the percentage of waste and its potential sources, arranging them according to priority.	•Brainstorming •Cause and Effect Map • SIPOC • Value Stream Mapping • Checklist
Do	1. Suggesting an appropriate solution based on the data. 2. Implementing the proposed solution.	Based on the problem analysis, the appropriate solution is determined; such as using a list to understand the desires and needs of the Hujaj of the agency, identifying their health status, finding an appropriate way to provide them with appropriate quantities of food throughout Hajj period and avoiding fatty foods that cause indigestion, etc. (attached with the research are practical solutions for the application of Deming Cycle and Lean Methodology)	• S5 • Kaizen
Check	1.Evaluation of the results achieved. 2. Ensuring that goal has been reached.	At this stage, a comparison is made between previous and current data, such as the percentage of Hujaj satisfaction before and after proposing and implementing the suggested solution, as	

		well as the percentage of wasted food before and after implementing the suggested solution.	• Brainstorming • Checklist
Act	1- If the results are satisfactory, the solution shall be circulated, and if they are not satisfactory, the plan shall be reexamined.	Based on the previous step, if the solution is satisfactory and achieved tangible results, such as increasing the Hujaj satisfaction with the food provided by 50% or more, as well as reducing the percentage of wasted food by 50% or more, then the solution will be circulated to other agencies, learning from the mistakes that occurred during implementation, if any, and working on avoiding them in next times, till reaching the maximum percentage of Hujaj satisfaction and the minimum percentage of waste.	• Brainstorming • Cause and Effect Map

Appendix 1: Proposal of Pilgrims' questionnaire to obtain basic data required for food management by the agencies. (Should be translated according to the language spoken by the Pilgrims)

Pilgrims questionnaire

(To meet the nutritional needs of Pilgrims during Hajj and Umrah)

Dear Pilgrims

The Agency is keen to provide you with the proper nutrition appropriate during Hajj period, so please answer the following questionnaire for us to know your nutritional needs and health status to provide you with the appropriate diet that maintain your health.

Thank you and appreciate your cooperation

Agency Name: _____

1. **Gender:** ☐ Male ☐ Female
2. **Have you performed Hajj before?** ☐ Yes ☐ No
   If (Yes), how long has passed since that Hajj?
   ☐ One Year ☐ Two Years ☐ Three Years ☐ Four and more years
3. **Age (Y)**
   ☐ Children (2: 12 Y) ☐ Teenagers (13: 21 Y) ☐ Adult (22: 60 Y) ☐ Elderly (60 and above)
4. **Do you suffer from any chronic diseases that require special diet?**
   ☐ Diabetes
   ☐ Hypertension
   ☐ Ulcers
   ☐ Duodenal ulcer
   ☐ Colon problems
   ☐ Digestive problems
   ☐ Gallbladder and liver disease
   ☐ Gluten sensitivity
   ☐ Food allergy (Please mention) .....
   ☐ Other diseases (Please mention) .....
5. What are your favorite cooking methods?
   ☐ Grilling ☐ Boiling ☐ Steaming ☐ Roasting ☐ Saucing **6.**

What are your favorite types of food for breakfast and dinner meals?

☐ Beans and chickpeas ☐ Cheeses ☐ Eggs
☐ Croissants and pies ☐ Fruits ☐ Bread
☐ Fresh vegetables ☐ Jam and halva ☐ Others .....

7. What are your favorite types of food for lunch meal?

☐ Red meat ☐ Poultry ☐ Fresh vegetables
☐ Cooked vegetables ☐ Fruits ☐ Bread ☐
Rice and pasta ☐ Salads
☐ Other choices.....

Appendix 2: Lean Methodology application on food management by Hajj and Umrah travel agencies				
	Plan	Do	Check	Act
Target	•Defining the problem. •Analyzing the problem based on gathered information.	•Suggesting an appropriate solution based on data. • Implementing the proposed solution.	•Evaluating the results achieved. •Ensuring that the target has been reached.	If the results are satisfactory, the solution shall be circulated and if they are not satisfactory, the plan shall be re-examined.
Methodology	• Using attached questionnaire to spot the actual needs of the Hujaj belonging to the agency •Brainstorming. •Cause and Effect Map. • SIPOC • VSM • Checklist • 5 Why.	•S5 (Sort, Set in Order, Shine, Standardize, Sustain) • Kaizen	• Map of operations • Brainstorming • Checklist	• Brainstorming • Cause and Effect Map
Methodology Guide	**1.Brainstorming:** Collective thinking and attempt to come up with creative solution ideas to find ato a specific problem, thinking about the pros and cons of the proposed solutions and choosing the best. **2.Cause and Impact Map:** Or the "Fishbone", is used to identify the potential causes of any problem by linking the effective causes and the resulting impact, with a focus on the causes in order to develop ideas and propose appropriate solutions to improve service delivery. **3.SIPOC:** One of the operations design tools by determining and identifying the suppliers, inputs, processes, outputs and customers, thus setting standards and indicators to measure them, spot the defects and determine who is responsible for their occurrence. **4.Value Stream Mapping (VSM):** A chart used to describe the process flow and the steps and procedures that the service goes through, which helps to clarify the operations accurately and thus the possibility of proposing amendments and improvements in service provision. **5.Checklist:** Used to collect and classify data into groups with similar characteristics, which contributes to facilitating data analysis and thus identifying the problem and taking appropriate action to solve it. **6.5 Why:** A tool used to find out the root cause of a problem or defect occurrence by asking several questions starting with "Why". **7.S5 (Sort, Set in Order, Shine, Standardize, Sustain):** A tool aimed at arranging the workplace, to ensure that the work is performed efficiently, effectively, and safely. This system focuses on putting everything in its designated place, maintaining the cleanliness of the workplace and making it easier for people to do their jobs without wasting time or being at risk. **8.Kaizen:** A Japanese methodology that aims to get rid of waste at work, with a view to achieve continuous improvement. It includes all employees without exception and urges them to perform simple daily changes to get rid of waste of all kinds, which leads to improving work in the long run.			

By implementing Deming Cycle with Lean Methodology, the basic problems of the research will be solved; the failure to meet the food needs suitable for Pilgrims, the great waste of food and its consequences, such as environment pollution, damaging the general view. Besides, solving other problems such as the wasted place for storing surplus food, transporting waste to and from the camps, as well as the wasted efforts of the agency workers in providing quantities of food more than required, reducing pollution resulting from excess food

that is not disposed of properly, in addition to reducing waste in the human resources, materials and devices used in preparing food. Finally, reducing the funds wasted in processing, transporting, cooling, heating, and preparing food.

CONCLUSION

From the obtained results to activate the role of research in saving the waste of food provided to Pilgrims, a form has been designed that the Agencies are required to distribute to their Pilgrims before time of Hajj to undertake the required preparations. Such forms are to be filled out by each Pilgrims including food preferences, preferred cooking methods, food habits, the usual quantities of food consumed, health conditions, and food sensitively. This information could be used in application of Lean Methodology in the food management and the reduce its waste.

Therefore, it is recommended that Hajj Agencies should have a nutrition supervisor to determine the nutritional needs of the Pilgrims, their favorite food considering their health status. Besides, supervise safety and suitable food preparation stages, determine the quantities to be served, in a manner that achieves maximum benefit for Pilgrims with avoiding the waste as 'What cannot be measured cannot be improved'. In addition, paying attention to the suitable re-preservation and repackaging methods of edible food in each Hajj Agency in a good way, to be redistributed to the needy. Increasing investments about the need to rationalize food consumption and reduce waste, using screens to be scattered all over the holy places and the indicative panels on the roads.

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Double Gene Targeting in Multiplex PCR for Discriminating Raw Meat of Cow, Buffalo, Goat and Sheep Species

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ABSTRACT

Meat is used as nutritious food, as it provides a sufficient amount of protein, vitamins, and minerals to be used for human consumption. In the present time, it is notable that meat and meat-related products are high in demand, consequent to the rise in the human population and their disposable income. Nowadays, consumers are concerned about the adulteration of meat or meat products and request accurate marking; therefore, to identify the meat correctly, proper checks are needed so that accurate identification can be made in a short time and the customer can be assured of the right meat. This study was carried out to differentiate Cow, Buffalo, Goat and Sheep meat by targeting 12S rRNA and cytochrome b gene. Mitochondrial Analysis of DNA was the most frequently used DNA because of its highly conserved sequences in various organism species. Two set of primers were utilized to amplify the 12S rRNA gene of cow and buffalo and another two set of primers were utilized to amplify the cyt b gene of goat and sheep. Initially species specificity of these primers was tested by running a conventional PCR using a pair of primers, the forward CYTCFP (for goat and sheep), 12SCFP (for cow and buffalo) and the reverse primers which were species specific. The primers in the multiplex PCR amplified target sequences at the ability comparable to ordinary PCR. Amplified PCR products for four species ranged from 159 to 501bp (Cow 501bp, Buffalo 229bp, Goat159bp and Sheep 336 bp) respectively. It was observed that from amplified PCR product of cyt b gene and 12S rRNA gene by utilizing the protocol of conventional PCR and could be obtained a species-specific band from isolated genomic DNA from four meat species. Multiplex PCR was created and can be used for synchronous recognition of numerous species origin in meat by cyt b, and 12S rRNA gene-derived species-specific primers.

KEY WORDS: MULTIPLEX PCR, SPECIES SPECIFIC MEAT DETECTION, ADULTERATION.

INTRODUCTION

From the nourishing perspective, meat is a rich source of essential amino acids and provides some amount of minerals also. Organ part of meat like liver is a good source of nicotinic acid, Vitamin B1, and Vitamin A. Scientific exploration is still in progress for better understanding of the contradictions of variation in different animal breeds and species for good health. It is very apparent from the past research that meat having less connective tissue is probably going to have low scores of absorption and digestion. For decades, meat adulteration has been taken for granted as inevitable. The main reason for this is an excess of demand over supply, favoring the seller over the buyer (Li et al., 2019).

In present time food adulteration involving animal species is very common to the mixing of cow, buffalo, goat and sheep meat. Due to religious, health and economic value it is very important to identified origin of meat product

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(Shi et al., 2019). In current scenario, every person wants to know exact source of meat which they are getting for consumption; so, it is important to inform consumer's choice, respect their lifestyle, religion, diet and health concerns. The price of meats from different species differs so substantially that meat vendors tend to misrepresent meats for economic gain. Buffalo are culturally and economically preferable meat having the large range of utilization in many countries. Indians prefer Sheep mutton because of their nutritious value, and avoid goat because of religious requirements. On the other hand, cow is totally unacceptable to the Hindus (Lin et al., 2019).

In earlier times, many morphological, protein, and lipid-based methods were used for the correct identification of animal meats or their associated food products, but these methods are not reliable due to the breakdown of the analyzed biomarker during food processing. Based on anatomical structures of various species of animals utilizing for meat generation, we can undoubtedly recognize the origin of meat species. If meat is in the lean, then the anatomical method is not a method for the specification of meat species (Song et al., 2019). Analytical methods are indispensable for labeling meat products because of the long time it takes, as this requires simple and rapid procedures so that precise labeling of meats or meat products can be easily done (Guo et al., 2019). Protein techniques are based on the expression of genes, and the immunoassay technique is used for detecting animal species in raw meat samples. But due to the possible similarity among antibodies in closely related meat species, these techniques are intense and followed by a lack of express to antibodies themselves, (Mansouri et al., 2020).

Food authenticity based on physical condition is either a tough task or impossible because of the damage in morphological parameters during processing and packaging (Taboada et al., 2014, Ali et al., 2015). PCR based techniques have shown great success in animal species identification due to its high level of specificity, sensitivity, accuracy and precision. PCR based detection techniques are reliable because this technique amplifies specific DNA targets from very less amount of sample. In present days an advance technique; PCR restriction fragment length polymorphism (PCR-RFLP) is very helpful in identifying meat authentication. It is identifying the meat species by cut the amplified PCR product at a specific position by using one or more restriction enzymes (Rashid et al., 2015). Using the cutting site variation that exists within a specific region of DNA, the differentiation of even closely related species is possible using a PCR-RFLP assay (Hsieh et al., 2016).

For reducing both time and cost Multiplex PCR technique with the use of species-specific primers are preferred since this technique offers multiple amplification in a single PCR (Qin et al., 2019). Multiplex PCR is a technique that includes the concurrent detection of various species. Both mitochondrial and genomic genes have been targeted for species detection by utilizing a multiplex PCR technique (Wang et al., 2019). Among the benefits of this technique are its efficiency, reliability, and sensitivity for mixed meat samples. In this regard, such advanced molecular methods like Multiplex PCR technique with the utilization of species-specific primer should be right-hand techniques for the detection of specific meat in a food product or unprocessed meat (Liu et al., 2019).

In the era of globalization and increased concern for animal food quality and safety, there is a great need to develop rapid, sensitive, specific and reproducible methods for authentication of meats (Cahyadi et al., 2019). Thus, this technique will also help to strengthen the growth and commercial potential of the nation's well-organized meat industry by creating awareness between traders and consumers about the adulteration of meat. Hence, this study is proposed to develop simple, rapid and reliable speciation techniques for authentication of meat species like cow-buffalo and goat-sheep.

MATERIAL AND METHODS

Sample collection and preservation: Raw and fresh meat samples of goat, sheep and buffalo were collected from different butchery of Lucknow, in clean and sterilized containers. For DNA isolation cow samples were taken from biopsy method because in UP, India cow slaughter is totally banned and illegal. Preserve the meat samples at -200C until used for further analysis.

DNA Extraction: Genomic DNA was isolated from the meat samples by Phenol-chloroform method as described by Sambrook and Russel (2001) with slight modifications. The tissue samples (75 mg) were cut into very small pieces or pulverized in liquid nitrogen and 10 volumes (w/v) of DNA lyses buffer (fresh meat) (pH 8.0) containing Ribonuclease-A at 100 µg/ml (20 µg/ml) was added and incubated at 37° C for 1 h. Proteinase-K solution (20 mg/ml) was added at 200 µg/ml and again incubated at 50°C for not less than 3 h or overnight. Equal volume of Tris-saturated phenol (equilibrated with 0.1 M Tris-Cl, pH 8.0) was added and the contents of the tubes were subjected to gently mixing end to end for 10 min and centrifuged at 6,500 RPM for 15 min.

The upper aqueous phase collected was washed twice with equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture. The upper phase was again collected in to a fresh tube containing 1/5 volume of 10 M ammonium acetate and double volume of absolute ethanol and was mixed well for precipitation of DNA. The mixture containing visible DNA threads was centrifuged at (10,000 RPM for 10 min). The DNA pellet was washed twice with 70 per cent alcohol by centrifugation (10,000 RPM for 5 min each), dried over a dry bath at 60° C and then dissolved in 1X TE (Tris-EDTA) buffer (50- 100 µl) or nuclease free water. Subsequently, the quality of DNA was checked on 1% agarose gel (Figure 4).

Primer Design: Mitochondrial sequences of cow, buffalo, goat and ship were downloaded from the NCBI database

and aligned using M-Coffee alignment program. The NCBI accession numbers of cow (Bos Taurus), buffalo (Bubalus bubalis), goat (*Capra hircus*) and sheep (Ovis aries) were respectively AF492351.1, KX758401.1, KY662383.1 and KP229295.1.Specific primer sets (sequences are provided in Table 1) with similar annealing temperatures were designed with Primer3 on the basis of cyt b (goat and sheep) and 12S rRNA (cow and buffalo) gene sequences. The specific primers were verified insilico by SnapGene software.

Table 1. Sequences of primers used for Multiplex PCR of cyt b and 12S rRNA gene

S. No	Name	Primer	Sequences (5'- 3' direction)	No. of Bases
1	12SCFP	F	GTGACAAAAATTAAG CCATAAACG	27
2	CORP	R	TTTTTATGTATCATAA TTACGCTTACTTTTT	31
3	BORP	R	GTGTGTCAGCTGTTA TAGAGTCACTTTCGT	30
4	CYTCFP	F	GACCTCCCAGCTCCATCAA ACATCTCATCTTGATGAAA	38
5	GRP	R	ATCTCGACAAATGT GAGTTACAGAGGAAAA	30
6	SRP	R	ATAGCCTATGAATGC TGTGGCTATTGT	27

Figure 1: Insilico Primer validation of Cow's species-specific cytochrome b gene.

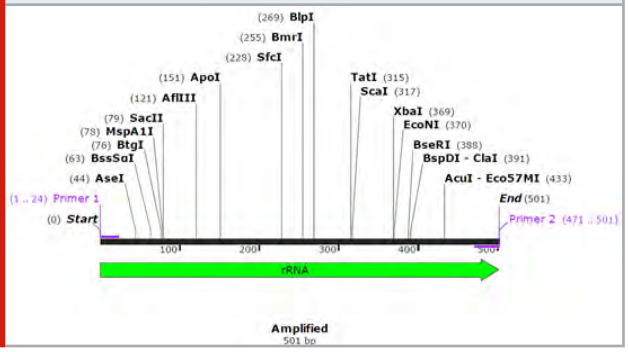
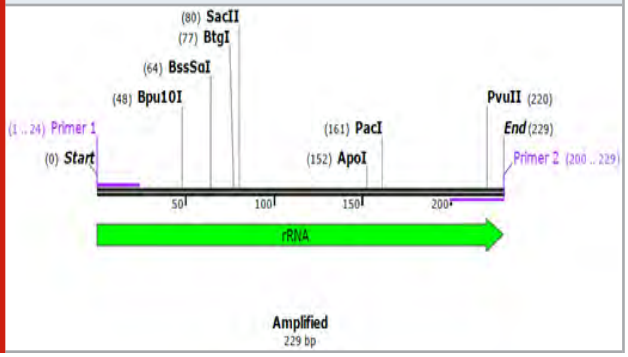


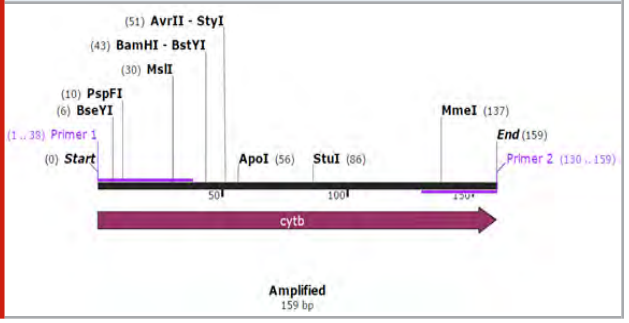
Figure 2: Insilico Primer validation of buffalo's species-specific cytochrome b gene.



Primer validation by In silico method: SnapGene tool (http://www.snapgene.com) was used for validating species specific primers, by using species specific sequences retrieved from NCBI for cytochrome b and 12S rRNA gene. The sequences of 12S rRNA gene of cow (Accession no. - AF492351.1) and buffalo (Accession no. - KX758401.1) and for cytochrome b gene of sheep (Accession no. - KP229295.1) and goat (Accession no. - KY662383.1) were retrieved from NCBI, used for validation of designed species specific primers. In silico primer validation results are given in the figure 1, figure 2, figure 3 and figure 4 for cow, buffalo, goat and sheep respectively. Primer sequence and target region on cytochrome b gene and 12S rRNA gene checked by TCOFEE (multiple sequence alignment tool) are showing in figure 5 and figure 6 respectively.

Primer Synthesis: Species specific designed primers were purchased from Micelles Life Sciences (P) Ltd. Initially primers were in desalted state, dilute the primers (in concentration 100pm/µl) with double distilled water as mentioned by manufacturer and stored at 20 0C for further analysis.

Figure 3: Insilico Primer validation of Goat's species-specific cytochrome b gene.



PCR amplification of the gene: For amplify 12S rRNA by conventional PCR, 0.5µl 12SCFP (12S r RNA common forward primer) and 0.5µl of reverse primer of cow and buffalo were used independently and for amplify cyt b gene by conventional PCR, 0.5 µM CYTCFP (Cytochrome common forward primer) and 0.5 µM of reverse primer of goat and sheep were used independently. 12SCFP was used as forward primer for cow and buffalo; and CYTCFP was used as forward primer for goat and sheep. After confirming all the species by using both gene primer individually, perform multiplex PCR by using multiplex PCR kit (Quiagen, Cat No.: 206143) by mixing all primers (conc. of each primer 0.5 µM) in single reaction with template DNA.

Figure 4: Insilico Primer validation of Sheep's species-specific cytochrome b gene.

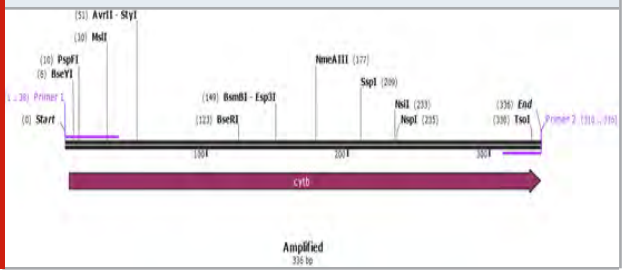


Figure 5: Primer sequence and target region on cytochrome b gene. CYTCFP indicates common forward primer for cytochrome gene. GRP and SRP indicate specific reverse primer of goat and sheep respectively.

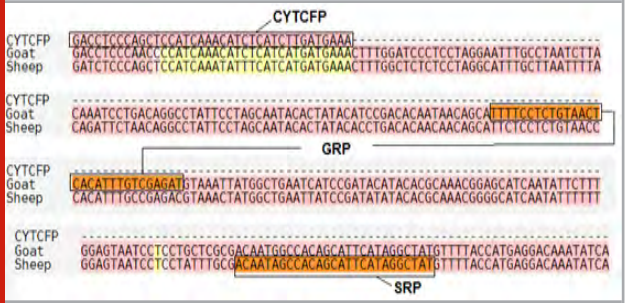
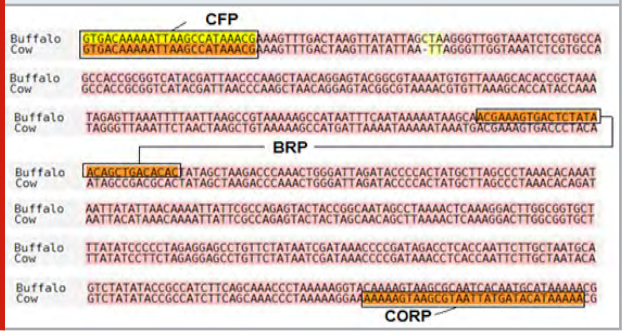


Figure 6: Primer sequence and target region on 12S rRNA gene. CFP indicates common forward primer for 12S rRNA gene. BRP and CORP indicates specific reverse primer of buffalo and cow respectively.



Firstly, mix all the primers in equal part and incorporate 4 µl in PCR reaction. Optimize the PCR reaction according to primer concentration. The PCR optimization used for 12S r RNA and cyt b gene consist of pre denaturation at 940C for the duration 5 min., denaturation (40 cycle) at 940C for the duration 30 sec., annealing (40 cycle) at 600C for 30 sec., elongation (40cycle) at 720C for 30 sec., and final elongation at 720C for 10 min.

Gel visualization of amplified PCR Product: Amplified DNA sample were separated by 1% agarose gel electrophoresis and each sample produced a characteristic band pattern at constant voltage 90V for 45 minutes. After running the gel, DNA bands with separate size were visualized under UV light and documented by gel doc.

RESULTS AND DISCUSSION

Meat is used as a nutritious food because it provides a sufficient amount of protein, vitamins, and minerals to be used for human consumption. In the present time, notably, meat and meat-related products are high in demand, consequent to the rise in the human population and their disposable income. From a nutritional point of view, meat is an excellent source of essential amino acids and also provides some amount of minerals, which are very important for the growth of the human body. Organ parts like liver is a good source of nicotinic acid, Vitamin B1 and Vitamin A. Scientific exploration is still in progress for better understanding of the contradictions of variation in different animal breeds and species for good health (Li et al., 2019).

Adulteration of meat species is a vast and serious problem around the world, which constitutes economic fraud in violation of food labeling laws that raise concerns about food security ethically and religiously (Hsieh et al., 2016). Therefore, to secure food security to the public as well as all over the world, fast and accurate methods for detecting the species of meat should be regulated by which quality control can be done (Manso uri et al., 2020). Species of cooked meat are difficult to identify because heat transmission during cooking is widespread, leading to changes within the meat tissue (Shi et al., 2019). Despite this change, short nucleic acid sequences are capable of surviving their processes even at maximum cooking temperature, allowing it to be employed to detect meat species (Cahyadi et al., 2019). Numerous researchers have shown interest in creating analytical methods and distinctive techniques for identification of specific meat species or their processed products, including a wide range of degraded and prepared materials that were predominantly based on estimating either DNA or protein (Qin et al., 2019).

Nucleic acid-based techniques popularly known as molecular techniques have been preferred over other techniques used in recent years because of the DNA composed of the organism's complete genetic information of a person (Liu et al., 2019). The nucleic acid-based analysis has been widely used in many fields, and these techniques became very popular for the discrimination

and detection of feed or food adulteration due to the stable properties of DNA (Song et al., 2019). (Taboada et al., 2014) reported that the presence and characteristics of proteins depend on tissue type, and at the same time, he also clarified that DNA is present in all cells and is almost identical and allows differentiation in closely related species by its unique variability and diversity. In the category of DNA based techniques, PCR is the most utilized, straightforward, efficient, sensitive, and specific method that can identify the species of origin presented to various processing circumstances (Manso uri et al., 2020).

Both mitochondrial, as well as nuclear DNA, have been used for meat differentiation; but mitochondrial analysis of DNA has been more frequently used method, because of its highly conserved sequences in various organism species (Hsieh et al., 2016). Mitochondria are the powerhouse of the cell, which have small filamentous and granular intracellular bodies. In comparison to other molecular markers like nuclear DNA, sequences of mtDNA (mitochondrial DNA) provide a lot of advantages. Due to the low number copy of DNA sequences, identification of nucleus DNA might be low scoring. In comparison to nucleus DNA, mitochondrial genes evolve a lot quicker, and, in this manner, mt DNA holds more sequence diversity variety, encouraging the distinguishing proof of phylogenetically related meat species (Qin et al., 2019).

For this study, genomic DNA was isolated from the meat samples by Phenol-chloroform method as described by Sambrook and Russel (2001) with slight modifications. In order to know the quality and quantity of DNA from agarose gel (Figure 7) and spectrophotometer, DNA was eluted in equal amounts, and the same amount of DNA was loaded into DNA gel. On the basis of DNA gel electrophoresis result and spectrophotometer reading, maximum DNA yield was obtained with the Sambrook and Russel standard method. For this work, two different sets of primers with similar annealing temperatures were designed using Primer3. Both sets contained a common forward primer that could amplify the genes of all species used in this work, and different special reverse primers were designed for each of the species.

The first set was specific for cyt b gene (goat and sheep), and the second set was for 12S rRNA genes (cow and buffalo). Mitochondrial sequences used to design the primer were downloaded from the NCBI database and aligned using M-Coffee alignment program. The NCBI accession numbers of cow (Bos Taurus), buffalo (Bubalus bubalis), goat (*Capra hircus*), and sheep (Ovisaries) were respectively AF492351.1, KX758401.1, KY662383.1, and KP229295.1. The specific primers were verified in-silico by SnapGene software. In the present study, to identify meat species, mt DNA of cyt b gene fragments were amplified by conventional PCR from genomic DNA of meat sample using a common forward primer, a species-specific primer for goat and sheep. All autonomous PCRs were successfully amplified and displayed the expected size fragment. Amplicons of size 159 bp (goat) and 336 bp (sheep) were observed when amplified products were run on agarose gel and size of amplicons was confirmed by running parallel a 100 bp DNA marker (Figure 8).

Figure 7: Agarose gel Electrophoresis of genomic DNA isolated from meat samples, where Lane M, 1, 2, 3 4 and 5 represent Marker (1kb), cow, buffalo, goat, and sheep respectively.

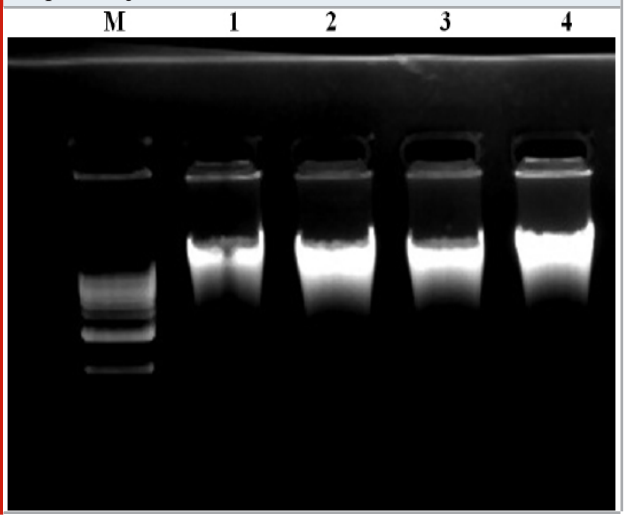
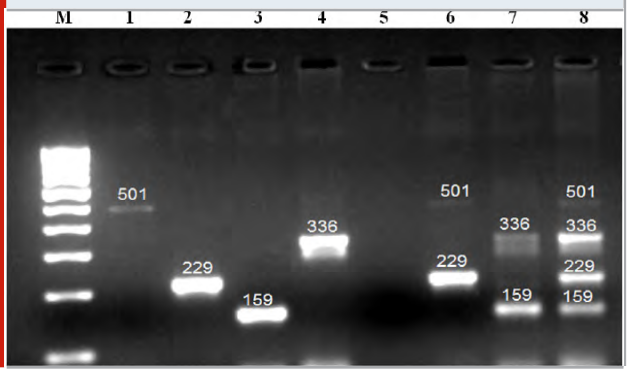


Figure 8: Agarose gel electrophoresis of PCR products amplified with Multiplex-PCR for the fragments of 12S r RNA (Cow and Buffalo) and Cyt b (Goat and Sheep) gene. Lane 1 represents product from Cow (501bp), Lane2 represent product from Buffalo (229bp), lane 3represent product from Goat (159bp), lane 4 represent the product from Sheep (336bp), Lane 5 represents the Negative control, Lane 6 represents the cow buffalo duplex ,Lane 7 represents Goat and Sheep Duplex, Lane 8 represents the Multiplex PCR products of Cow, Buffalo, Goat and Sheep. M – 100bp DNA ladder.



In the present study, to identify meat species, mt DNA of 12S rRNA gene fragments were amplified by conventional PCR from the genomic DNA of meat sample using a common forward primer, species-specific primer for cow and buffalo. All autonomous PCRs were successfully amplified and displayed the expected size fragment. Amplicons of size 501 bp (cow) and 229 bp (buffalo) were observed when amplified products were run on an agarose gel, and size of amplicons was confirmed by running parallel a 100 bp DNA marker (Figure 8).

Multiplex PCR is a technique that includes the concurrent detection of various species. Both mitochondrial and genomic genes have been targeted for species detection by utilizing a multiplex PCR technique (Qin et al., 2019). Among the benefits of this technique are its efficiency, reliability, and sensitivity for mixed meat samples. For identification of poultry residuals (Song et al., 2019) utilized this technique by using mitochondrial 16S rRNA and 12S rRNA as a molecular marker.

In the present study after confirming the species specificity of each primer independently by conventional PCR, a multiplex PCR was prepared by mixing all primers in a single microcentrifuge tube. In multiplex PCR, a primer cocktail containing the designed common primers and species-specific primer was used with DNA extracted from the meat samples. After successful completion of the multiplex PCR reaction, when the amplified product was run on agarose gel and size of amplicons was confirmed by running parallel a 100 bp DNA marker, the same expected size bands were seen as shown in conventional PCR for specific species, no difference was found between them.

After studying the electrophoretic pattern, it became clear that there was a complete absence of any cross-reaction in this work, and in fact, only species-specific bands were clearly visible. To differentiate the meat mixture and show the applicability of multiplex PCR on mixed meat, we adapted multiplex PCR under the same conditions in which simplex PCR was run. The size of the PCR products of the target species was expected, and no additional fragments were displayed. Studying this result made it clear that species-specific primers amplified only a particular size fragment from a target species, and amplification of this primer was not possible with any other species. The PCR product showed species-specific DNA fragments of 159, 336, 501, and 229 bp from goat, sheep, cow and buffalo meat respectively (Figure 8). All PCR amplicons resulting from these particular reverse primers had a size of between 159–501bp, thus using this technology, the species of all animals used in this task can be detected very easily by running their PCR amplicons in the same agarose gel.

CONCLUSION

Meat positions among one of the most nutritious and vitality rich sustenance item, used by the people to satisfy their standard body necessities. It is considered significant in keeping up a better and balanced eating routine, which is basic in achieving ideal human development and advancement. In the present time, notably, meat and meat-related products are high in demand, consequent to the rise in the human population and their disposable income. In the present study, after confirming the species specificity of each primer independently by conventional PCR, a multiplex PCR was prepared by mixing all primers in a single microcentrifuge tube. In multiplex PCR, a primer cocktail containing the designed common primers and species-specific primer was used with DNA extracted from the meat samples. After successful completion of the multiplex PCR reaction, when the amplified product was run on an agarose gel and the size of amplicons was confirmed by running parallel a 100 bp DNA marker, the same expected size bands were seen as shown in conventional PCR for specific species, no difference was found between them. After studying the electrophoretic pattern, it became clear that there was a complete absence of any cross-reaction in this work.

In fact, only species-specific bands were clearly visible. In order to differentiate the meat mixture and show the applicability of multiplex PCR on mixed meat, we optimized multiplex PCR in the same conditions as for simplex PCR. The size of the PCR products of the target species was expected, and no additional fragments were displayed. This result showed that, as species-specific primers amplified only one size fragment from a target species, amplification of this primer was not possible with any other species. The PCR product showed species-specific DNA fragments of 159, 336, 501, and 229 bp from goat, sheep, cow and buffalo meat respectively. In conclusion, it was observed that all PCR amplicons resulting from these particular reverse primers had a size of between 159-501bp, thus using this technology, the species of all animals used in this task can be detected very easily by running their PCR amplicons in the same agarose gel.

Conflict of Interests: There is no conflict of interests between the authors.

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Development and Evaluation of Salubrious Soup Mix Incorporated with Ridge Gourd Peel Powder

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ABSTRACT

A surplus amount of waste is engendered along with the entire gamut of food production industries in the form of skin or peel, which can be utilized for human consumption after suitable processing. Ridge gourd peel is an edible bio waste which is not used due to high dietary fiber content and a rough texture. The peel is healthy and contains good amount of fiber, vitamins, antioxidant and minerals. Copious types of processed food products are available in the markets, but the majority of consumers prefer instant products only. Based on this inclination, an attempt has been made to develop ridge gourd peel powder incorporated soup mix. The different variations of soup mix were prepared by using ridge gourd peel powder, spice powder and thickening agents. The sensory evaluations were done in all variations of the prepared soup mixes, and nutritional analysis and antioxidant property for the accepted variation of the final soup mix were ascertained. The drying temperature for the preparation of ridge gourd peel has been calculated through proximate analysis. It was observed that the ridge gourd peel powder incorporated instant soup mix was rich in fiber, vitamin C, calcium, potassium and iron and significantly reduced in carbohydrate, protein, fat and sodium content compared to control soup mix. The cost calculation for 100g of developed (Ridge gourd peel powder added) soup mix was 35.24 rupees. It was evident that the prepared soup mix was more economical and affordable when compared with commercial soup mixes available in the market.

KEY WORDS: SENSORY EVALUATION, SOUP MIX, VALUE ADDED, VEGETABLE PEEL POWDER.

INTRODUCTION

The Ridge Gourd is a popular vegetable in the Asian, African and the Arabic countries. The vegetable is popular in India, China and Vietnam. In Tamil Nadu, the ridge gourd is called Peerkangai. In Kerala, it is called Peechinga. Ridge gourd (*Luffaacutangula* L. Roxb), belongs to the genus Luffa, family Cucurbitaceae. It is popularly called as an angled gourd, angled loofah. Luffa is also known as Patola (Filipinos), angled or ribbed Luffa, silk gourd, dishcloth gourd, silk squash, and Chinese okra, Sin qua, etc. (Jaysingrao and Sunil 2012). Ridge gourd acts as an appetizer, and contains a good amount of fiber, vitamins. Ridge gourd has a sweet taste, cooling in nature and easy to digest. They form a low -calorie diet, hence considered good for diabetes. Both the soft pulp and skin of ridge gourd are used in making various recipes, especially chutneys in South Indian cuisine. (Manikandaselvi et al., 2016). Ridge gourd peel has high nutrients value and is often called a nutrition powerhouse because of its affluent and varied nutrient contents. It is also rich in vitamin C, flavonoids, calcium, potassium, sodium and essential amino acids. The peel contains glycerides of palmitic, stearic and myristic acids (Kandoliya et al., 2016, Vassilios et al., 2019).

Ridge gourd peel powders, as well as their various solvent fractions, were evaluated for anti-oxygenic activity using different methods. Ridge gourd peel powders at 2% level and their ethanol/water-soluble extracts exhibited intense

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anti-oxygenic activity in stored at 37˚C. Ridge gourd peel powders, as well as their extracts, were evaluated for their anti-oxygenic activity using linoleic acid peroxidation, β-carotene-linoleic acid bleaching methods. Ethanol/ water extracts from ridge gourd peel showed highest anti-oxygenic activity followed by water extracts, while the petroleum ether extract showed moderate anti-oxygenic activity. The phenolic compounds may contribute directly to the antioxidant action; therefore, it is necessary to investigate total phenolic content (Swetha and Muthukumar 2016). Ridge gourd peel powder and its extracts showed slightly higher anti-oxygenic activity than ridge gourd pulp powder and its extracts. It may be attributed to the presence of higher amounts of phenolics and flavonoids, which have been reported as potential antioxidants (Vyas et al., 2015). The objective of this study aimed to reduce the wastage and extend the usage of ridge gourd peel by developing a nutritious value-added soup mix.

MATERIAL AND METHODS

The ingredients required for the preparation of ridge gourd peel powder incorporated soup mix viz. fresh ridge gourd (Luffaacutangula), and ingredients for the development of spice powders and thickening agents were procured from the local supermarket, Krishnagiri, Tamil Nadu, India. The ridge gourds were washed thoroughly to take away of debris present on the surface of the skin and peel the skin and cut into medium-sized strips. The peels were shadow dried for 3 to 4 days. The dried peels were then powdered, sieved and packed in laminated aluminium foil pouches. Other Spice mix ingredients were dehydrated at 60°C, tomato dried at 80°Cand dried and powdered thickening agents were packed in aluminium foil pouches. Three different variations of soup mixes were prepared by using ridge gourd peel powder, spice mix and thickening agents. Ridge gourd peel powder soup mixes were replicated three times. The developed soup mixes were subjected to sensory analysis and accepted variation of soup mix was then analyzed nutritional composition and antioxidant activity.

RESULTS AND DISCUSSION

Development of the ridge gourd peel powder incorporated soup mixes: The soup mix blended with ridge gourd peel powder in different variations viz. V1, V2 and V3 and other spice mix and thickening agents also measured in different quantity and added in variations separately. The composition of ingredients used for developing soup mixes of different variations are shown in Table – 1.

Sensory evaluation of the ridge gourd peel powder incorporated soup mixes: Sensory evaluation was done for all the formulated ridge gourd peel powder incorporated soup mixes. By using these soup mixes, different variations of soups were prepared, and sensory evaluation was done in all the developed variations of prepared soups by semi- trained panel members using 9 points hedonic rating scale.

The results of the above table revealed that the mean score obtained for colour of V1 and V2 were found to be maximum (8.15 ± 0.14 and 6.12 ± 0.22) than control and V3. Mean score of texture was high (7.22 ± 0.54) in V1 compared to control and other variations. The results revealed that the mean score obtained for the flavour of V1 was found to be superior (8.37 ± 0.22) compared to control and other variations. Variation I had the maximum mean scores for taste (7.22 ± 0.47) compared to control and other variations. The overall acceptability was highly acknowledged in Variation I (6.13 ± 0.81) and variation II (6.41 ± 0.53). Based on the overall result, Variation 1 was highly accepted and selected for further analysis.

Table 1. Composition of ingredients for ridge gourd peel powder incorporated soup mix

Ingredients (gm)	Variation -1(g)	Variation-2 (g)	Variation-3(g)
Ridge gourd peel powder	18	25	31
Spice mix:			
Onion powder	5	6	7
Garlic powder	5	6	7
Tomato powder	5	6	7
Mint powder	5	6	7
Coriander powder	8	8	7
Cumin powder	10	10	7
Green chilli powder	5	6	7
Table salt	2	2	2
Spice mix	45	50	52
Thickening agent:			
Corn flour	18	12	7
Green gram flour	19	13	10
Thickening agent	37	25	17

Table 2. Statistical Analysis of Sensory Evaluation of the Developed Soup Mixes

Sensory attributes	Control	Variation I	Variation II	Variation III
Appearance	3.13 ± 0.64^{a}	6.13 ± 0.72^{a}	6.22 ± 0.51^{ab}	5.12 ± 0.71^{cd}
Color	3.21± 0.64^{ac}	8.15 ± 0.14^{cd}	6.12 ± 0.22^{a}	4.13 ± 0.34^{bc}
Texture	3.25 ± 0.46^{ab}	7.22 ± 0.54^{bc}	6.11 ± 0.71^{a}	3.13 ± 0.68^{a}
Flavour	3.42 ± 0.53^{a}	8.37 ± 0.22^{cd}	4.56 ± 0.27^{cd}	4.84 ± 0.89^{bc}
Taste	3.13± 0.64^{a}	7.22 ± 0.47^{bc}	5.22 ± 0.64^{c}	5.43 ± 0.51^{cd}
Overall Acceptability	3.38 ± 0.52^{bc}	6.13 ± 0.81^{a}	6.41 ± 0.53^{ab}	5.55 ± 0.22^{cd}

Values are mean ± SD of triplicate determination. Samples with different superscripts within the same column were significantly ($p \leq 0.05$) different.

Results on Duncan Multiple Range test showed that there was a significant difference (p- value 0.05) between control and the different variations of soup on colour, texture, flavour, mouthfeel, taste, and overall acceptability. Consumers are the judges of a product's fate and welfare in the market as their preference is of vital significance. Therefore, specific sensory properties of a product, along with its composition, may comprise a key for its uniqueness and support (Vassilios et al., 2019). Nutritional composition of accepted variation of the ridge gourd peel powder incorporated soup mix: After sensory evaluation, the panel members agreed and gave good remarks about the 18g ridge gourd peel powder incorporated (variation-1) soup mix. Based on the sensory evaluation of the developed soup mixes, 18% of ridge gourd peel powder included soup mix (Variation 1) was more acceptable. Hence the further analyses were done for variation 1 soup mixes. Nutritional compositions of accepted variation of the soup mix are shown in table 3.

Table 3. Nutritional Composition of Accepted Variation of the Soup Mix

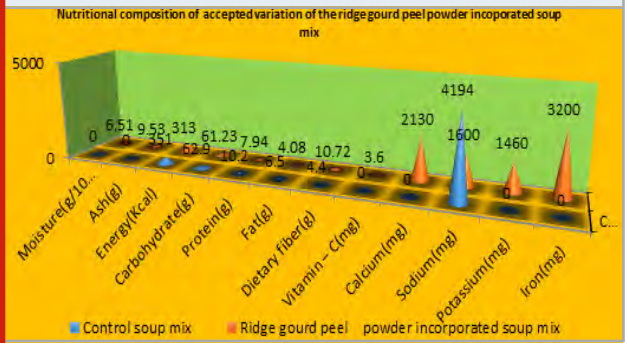
S. No	Nutrients	Control soup mix	Ridge gourd peel powder incorporated soup mix (variation-1)	Deficient or excess
1	Moisture(g/100g)	-	6.51	+6.51
2	Ash(g)	-	9.53	+9.53
3	Energy (Kcal)	351	313	-38
4	Carbohydrate(g)	62.9	61.23	-1.67
5	Protein(g)	10.2	7.94	-2.26
6	Fat(g)	6.5	4.08	-2.42
7	Dietary fiber(g)	4.4	10.72	+6.32
8	Vitamin – C(mg)	-	3.6	+3.6
9	Calcium(mg)	-	2130	+2130
10	Sodium(mg)	4194	1600	-2594
11	Potassium(mg)	-	1460	+1460
12	Iron(mg)	-	3200	+3200
13	Flavonoids	-	++	++

The moisture content of the ridge gourd peel powder incorporated soup mix was 6.51g/100g, and the ash content was 9.53g, and control soup mix had no moisture and ash content. Compared to the control soup mix, the energy and carbohydrate content were reduced in the accepted variation of the developed soup mix. There is a slight change in the protein, fat content between the ridge gourd peel powders incorporated soup mix and control soup mix. The dietary fiber content of the ridge gourd peel powder incorporated soup mix was 10.72g/100g which is higher than the control, as ridge gourd peel contains high fiber content which will aid digestion.

The vitamin C content of the ridge gourd peel powder soup mix was 3.6 mg/100g, but no vitamin C found in control soup mix. Calcium in developed soup mix was 2130 mg, but control soup mix had no calcium. The presence of high calcium content helps to improve bone health. The potassium content of the ridge gourd peel powder incorporated soup mix was 1460 mg/100g, but no potassium was present in control soup mix. The high potassium content helps to reduce Hypertension. The Iron content of the ridge gourd peel powder incorporated soup mix was 3200 mg/100g but in control soup mix had no Iron content. Iron in soup mix helps

to improve hemoglobin level in blood. Flavonoids are qualitatively present in the developed ridge gourd peel powder incorporated soup mix which helps to enhance antioxidant activity.

Figure 1: Nutritional Composition of Accepted Variation of the Ridge Gourd Peel Powder Incorporated Soup Mix.



Cost calculation of accepted variation of the developed soup mix: The cost calculation for the production of 100g of the developed soup mix was Rs.35.24 by incorporating ridge gourd peel powder, spice mixes and thickening agents. It was evident that the prepared soup mix was more economical and affordable when compared with commercial soup mixes available in the market.

CONCLUSION

Ridge gourd is one of the nutritious vegetables gifted by nature to human beings. Ridge gourd peel is usually considered as the waste or byproduct of ridge gourd. Still, they are rich sources of nutrients, especially dietary fiber, vitamin-c, calcium, potassium, iron and flavonoids. This study concluded that the development of soup mix from ridge gourd peel powder is a stupendous value-added liquid food. The nutrients profile of the soup mix was also appealing from the health point of view. Ridge gourd peel consumption provides several health benefits, and it acts as a natural protector against diseases. The developed ridge gourd peel powder added soup mix is a novel one that holds good commercialization potential.

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Conflict of Interests: The authors declare that they have no conflict of interest.

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Academic Alienation Among Adolescents in Relation to Emotional Intelligence and Resilience

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ABSTRACT

Adolescence is a period characterized by diverse changes that may be physiological, social or emotional and inability to deal with these changes may lead to maladjustment and popping up of various psychological issues like academic alienation. Adolescents should be made capable of handling their emotions effectively so as to develop the capability to deal with psycho-social problems. Thus, it is the need of the hour to develop emotional intelligence and resilience among adolescents so that they can effectively deal with the psycho-social problem like academic alienation. Present study was undertaken to investigate academic alienation among adolescents in relation to emotional intelligence and resilience. A sample of 500 adolescents was selected randomly from ten districts of Punjab. Academic Alienation scale (2015) by Rita Animanga Emotional Intelligence Inventory (2018) EII-MM by Mangal and Mangal, The Resilience Scale (2009) by Wagnild and Young were used to collect the data. The findings of the present research divulge that emotional intelligence and resilience are important factors in reducing the level of academic alienation. The results reveal that there is a significant and negative relationship between academic alienation and emotional intelligence among adolescents. A significant and negative relationship was also found between academic alienation and resilience among adolescents. It is quite apparent from the regression model summary that emotional intelligence and resilience would contribute towards the prediction of academic alienation of adolescents both independently as well as conjointly. Emotional intelligence and resilience are the most significant and influential contributor in predicting academic alienation among adolescents.

KEY WORDS: ACADEMIC ALIENATION, EMOTIONAL INTELLIGENCE, RESILIENCE.

INTRODUCTION

Education aims at facilitating optimum development of students in all spheres. In this era of 21st century learning and understanding about self is as important as learning about the various school subjects. Here, schools and parents are playing an important role in making self-assured and competent individuals. Though education is an inevitable factor for economic and social growth, it is very essential for building human capabilities and employment opportunities. However, today's education system is creating a challenging environment for the students with the existence of computer, mobile, new technological innovations, career stability and suitable placement. In order to achieve their aspirations students are getting stressed and academic anxiety has become a part of student's life. Fear of failing in examination, to face social gathering much more can lead adolescents to academic anxiety. Sometimes it results in exam phobia or academic alienation; as a consequence, alarming rates of suicides, depression, co-occurred with sleeplessness,

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inability to make decisions, heart palpitation, low self-esteem, low academic performance and alienation among adolescents has become the major concern for schools, parents and higher authorities. There is a need to provide conducive environment and to develop coping strategies to help young minds for the development of emotional intelligence to overcome alienation (Bhullar and Mandeep, 2019).

Adolescence has been recognized as one of the crucial stages of revolutionary changes (psychological, mental, dramatic physical growth, emotional) creates turmoil in their minds. Ultimately this turmoil leads to stress, uneasiness and discomfort. Alienation in adolescents may intensify the stressful events and has been associated with academic alienation. Academic alienation is most pertinent challenges of modern education system and is related to the issues like loss of control on self-decision making, anxiety, rootlessness, isolation, meaninglessness, loss of belief and values, loss of interest in studies, fear of failure, low academic performance, sleeplessness, telling lies, refusal to go to school, lack of self-confidence, hiding the school performance card, drunkenness', smoking, drug addiction and interpersonal dishonesty. So, in today's highly competitive world, students face various academic issues too, sometimes they are unable to understand homework assignments, have inability to understand the subjects, lack of time management, expectations about high academic success etc. This changed scenario in the education system gave rise to stern competitive procedures for evaluating the students' aptitude, knowledge, ability and intelligence (Stoker and Perkin, 2014).

This excessive anxiety, stress and fear regarding examination is the solo reason for academic alienation, which is rather common among adolescents. Academic Alienation due to examination stress and test anxiety is pervasive problems of the modern educational processes (Forsyth 1986). There are also research evidences which suggest that academic achievement influences social behaviors and students' academic and emotional adjustment. According to attribution theory, failing students are more likely to ascribe their under achievement to external factors such as teachers or learning conditions (Weiner 1985; Zhou et al., 2010). Particularly external attributions result in negative affect and behavioral reactions close to school alienation (e.g., helplessness, a motivation, low school attendance and participation). As a consequence, students experience high degree of stress and anxiety before and during exams, test anxiety can actually impair learning and affect test performance. To Ensure the eminence of adolescents' educational outcomes, long-standing history in secondary schools remains the top priority of the policy makers (Stoker and Perkin, 2014).

However, students with their experiences in everyday school life, who are, at the same time, going through, the time of intense changes associated with pubertal development, are likely to deal with a combination of different stressors that may instigate the development of Academic alienation and hamper students' academic success (Mayer, 1997; Schunk and Meece , 2005). Along with these pressures that are brought to bear on them, students are almost inevitably confronted with the necessity to act productively in the learning environment from which they are alienated, fulfilling increasing demands of the education sector (Eccles et al. 2008; Yazzie-Mintz and McCormick 2012). Hence the question of coping up with test anxiety and academic stress by students using the famous strength called emotional intelligence needs to be answered (Yazzie-Mintz and McCormick 2012).

Academic stress needs to be managed by the strengths and powers the students have. Because Emotional intelligence is a type of social intelligence that involves one's ability to monitor one's own emotion as well as those of others, to discriminate among them and to use that information to guide one's thoughts and actions. Emotional intelligence includes the verbal and nonverbal appraisal and expression of emotion, effective regulation of emotion in the self and others, it promotes utilization of emotional content in problem solving. Emotional intelligence is essential for interpersonal and intrapersonal relationships at school, at home and at work (Brackett et al., 2011; Bar-On, 2014).

People with high emotional quotient are expected to progress more quickly through the designated abilities and to master more of them. It is the capacity to create positive outcomes which include joy, optimism, and success in school and life. A variety of researchers have engaged in research designed to examine and apply emotional intelligence constructs within academic and other learning settings (Brackett and Katella, 2007; Mayer et al., 2009; Bronzes and Militia, 2014). Secondly, American Psychological Association (2011) describes resilience as, one of the factors causing the reactions of individuals to be different. It is used in the sense of restoring to the previous situation, i.e. flexibility, and it is the process to be adaptive to every challenge of life (Bronzes and Militia, 2014).

Morinaj, Hadjar and Hascher (2020) have examined that longitudinal relationship between school alienation domains, namely alienation from learning, teachers, classmates, and academic achievement among secondary school students of grade 7 to grade 9 in Switzerland and Luxembourg. This data was collected from 403 students in the Swiss canton of Bern (t1: 44.3% male; Mage = 13.0 years [SD = .54]) and N = 387 secondary school students from Luxembourg (t1: 57.4% male; Mage = 12.7 years [SD = .64]), who completed three waves at grades 7–9. The significant gender differences in the Swiss sample were observed in regard to all SAL domains, with boys being more alienated from learning, teachers, and classmates than girls.

In the Luxembourgish sample, boys exhibited higher levels of alienation from learning at t3, alienation from teachers at t2 and t3, and alienation from classmates at t1. The results also revealed that girls outperformed boys

in terms of grades across all three waves in both the Swiss and Luxembourgish samples These findings suggest that lower academic achievement was associated with higher alienation from learning, teachers, and classmates at the subsequent grades. It may be that students in the Swiss sample are confronted with increasing achievement pressure, influencing students' emotional and cognitive evaluations of the school reality. (Alienation from classmates–academic achievement relation would depend on the quality of interpersonal relationships with peers and teachers in the classroom.

Another study conducted by Zahra et al. (2018), the researchers explored two groups of 60 girls (30 Turkish and 30 Persian) and 60 boys (30 Turkish and 30 Persian). The study sample was taken through multi-stage cluster sampling from among first-year Tabriz University students in Tabriz, Iran. All the participants of the study were between 18 to 22 years were asked to respond on an academic alienation questionnaire. Two-way ANOVA was applied and findings revealed that girls and Turkish speakers had higher levels of academic alienation compared to boys and Persian speakers. Further the interactional effects indicated that Turkish Girls demonstrated the highest levels of academic alienation, with no significant effects in the powerlessness dimension.

Hascher and Hadjar (2018) found that students may be alienated from school in general, but beyond that, they are likely to be or become alienated from specific aspects or domains of schooling such as learning, teachers, and classmates. For this, the term alienation describes the process of increasing distancing from certain objects in the school environment and is associated with decreasing enjoyment of school. They further concluded that "a specific set of negative attitudes towards social and academic domains of schooling comprising cognitive and affective elements. While the cognitive dimension relates to student appraisals of the school environment, the affective dimension relates to their feelings and emotions.

These negative emotions and feeling develop and change over time in terms of a state and can solidify into a disposition. In a similar vein, previous research has also found that students with a low level of school-related emotional exhaustion are more resilient and able to 'bounce back' from negative experiences (Sorkkila et al., 2020). Whereas, Himmati and Pirnya (2017), conducted a study on 329 undergraduate and graduate students of the University of Isfahan. In these five dimensions of academic alienation like powerlessness, meaninglessness, anomie, cynicism and social isolation were measured. And their findings show that the average of academic alienation of the students taken is slightly above the average and the degree of feeling of powerlessness and cynicism is more than the other aspects.

The results also indicate that interaction with professors, academic motivation and attitude towards other has a direct impact and satisfaction of major attitude towards future career and self-concept, emotional well-being has the direct impact on reducing student's academic alienation. Tome and collaborators (2016) conducted a study on 3869 Portuguese Adolescent students and found a trend of association between alienation, recession, adolescents well-being, felling of stranger, powerless and hopelessness. They noted that the feeling of unsatisfaction with life seemed to have a lot of influence to feelings of powerlessness, while the association between normlessness and the poorest relationship with family was equally high.

To be satisfied with life and to have a good relationship with family were important assets for adolescent's mental health and emotional well-being. Further they also concluded that the adolescents with increased social isolation and normlessness have more involvement in risk behavior and association between the alienation, wellbeing and quality of life was negative. Thus, apparently lesser the alienation, social isolation and normlessness problems, higher will be the well-being. Apparently, emotional intelligence can prevent maladjustment and perceived stress through enhancing resilience in the academic context (Romano et al., 2019).

A study was conducted on 200 adolescents of Government senior secondary schools of Ludhiana district of Punjab, India. Data was collected with alienation scale constructed by investigator in 2014 and emotional intelligence scale by Mangal and Mangal were used for the investigation. The findings of the study indicate that there is a significant and negative relationship between alienation and emotional intelligence among urban adolescents (Kaur and Singh, 2015). The word resilient is used for individuals who are able to make progress unexpectedly and show success in challenging conditions (one who can recuperate quickly), and "resilience" is used as personality trait of these individuals (Terzi, 2008). Being flexible does not mean not having any difficulty or not encountering any negative situation, but is the ability to recover successfully by going back to the previous situation in risky conditions, despite serious threats to adaptation and development (Masten, 2001; APA, 2011). According to Garmezy (1993) the problem encountered is interpreted as a dynamic process which involves a positive adaptation process in a negative situation such as stress and distress. Resilience focuses on the strengths of individuals and their ability to overcome negative situations by using their own sources (Seligman and Csikzentmihaly, 2000).

Venta, Amanda and Cassandra, Rivas et al. (2019) found that school engagements made a significant, positive contribution to mental health and resilience for youth above and beyond the effects of parental and peer attachment. Sakiz and Aftab (2019) conducted a study on 810 students' studying in vocational and non-vocational high schools in Turkey. Quantitative data was collected through student's records and questionnaires are analyzed via descriptive, correlation and regression analysis and tests of difference (ANOVA). The results

of the study found that academic achievement and psychological resilience were significantly related and they changed based on socio demographic factors, namely income level and school type. More over psychological resilience has a significant mediating effect between academic achievement and socio demographic factors. Yuan, Zhang and Fu's (2017) explained the predictive role of thinking style for academic stress coping among secondary school students in grade 7 through 12 from mainland China. This study revealed a significant predictive power for academic stress-coping strategies beyond age and gender. The students who think more creatively are more sophisticated and cognitive in process information to have better problem-solving skills were more strategic in making plans to solve the problems.

Kim ,Yang Hou and Gonzalez(2016) found that adolescents with a strong sense of alienation from parents or low resilience (a) experienced more burden or less efficacy in translating and (b) were more susceptible to the detrimental effects of feeling a sense of burden and the beneficial effects of experiencing a sense of efficacy, as measured by depressive symptoms. The sample of the study comprised 557 adolescent language brokers (M age = 12.96) in Mexican-American families. Ifeagwazi, Chukwuorji and Zacchaeus (2015) described that interpersonal alienation, political alienation and socio-economic alienation were positively associated with psychological distress while resilience was negatively related to psychological distress.

Psychological distress was also predicted by alienation and resilience. Resilience neither moderated the relationship of interpersonal alienation and psychological distress nor political alienation and psychological distress but the relationship between socio-economic alienation and psychological distress was moderated by resilience. It was further concluded that initiation of resilience building program as a form of cognitive-behavioral and existential interventions may buffer the negative relationship of alienation to psychological distress. The moderator role of resilience on the relationship of the three facets of alienation and psychological wellbeing was also investigated.

A study was conducted to investigate the role of trait emotional intelligence in preventing students' school burnout directly and indirectly via anxiety and academic resilience. A sample of 1235 high school students (962 females and 273 males), ranging in age between 13 and 17 years was taken, (mean = 15.46; stand deviation = 1.22). Thus, Structural equation modelling revealed a strong indirect effect of emotional intelligence on school burnout, mediated via anxiety and resilience. Overall, students with high emotional intelligence were less likely to experience school anxiety and more likely to exhibit resilience which, in turn, reduced school burnout risk (Caterina et al., 2020). The study of the above quoted research studies relating to academic alienation in relation to emotional intelligence and resilience reveal that these variables have been studied more in western countries as compared to India. The review suggested that the studies on academic alienation in relation to emotional intelligence and resilience await empirical investigations. When adolescents are able to count on personal resources, such as resilience, they are in a much better position to overcome acute or chronic adversities that can compromise their academic career (Cassidy, 2015; May, and Regueiro, 2018; Bouer, 2020).

Operational Definitions of the terms used - Academic Alienation- It can be defined as certain perception and feeling of disorientation and disinterest that some students have about themselves and about one or more aspects of their social life and academic performance which in turn leads students to high drop-out rates and academic anxiety. It includes six dimensions i.e. Lack of interest in study, irresponsible attitude of teacher, monopoly of teacher, unsupportive environment, mismanagement and unable to express (Rani, 2015).

Emotional Intelligence: "Emotional Intelligence is the unitary ability related to independent of standard Intelligence helpful in knowing, feeling and judging emotions in close co-operation with one's thinking process to behave in a proper way for the ultimate realize of the happiness and welfare of the self in tune with four areas or aspects of emotional intelligence namely, Intra-personal Awareness(knowing about one's own emotions) Inter-personal Awareness (Knowing about others emotions) Intra-personal Management (Managing one's own emotions) and Inter-personal Management (Managing others emotions)" (Mangal and Mangal, 2018)

Resilience: Resilience is "the process of, capacity for, or outcome of, successful adaption despite challenging or threatening circumstances, it's an ability to recover from adversity and as a positive personality characteristic that enhance individual adaption and moderates the negative effects of stress" (Wagnild and Young, 1993).

Adolescence: Adolescence is that span of years during which boys and girls move from childhood to adulthood, This stage of adolescence is described as a stage of turbulence a 'period of stress and storm' of emotional in stability and confused period of human life, in which adolescent's perception of school, their academic successes and failures affect their overall sense of self. Adolescence is a transitional stage from childhood to adulthood and is a time of major changes in all area of functioning. Children and adolescents can experience various life stresses ranging from catastrophic or traumatic life events persistent strain and daily hassles. Academic matters are the most important sources of chronic and sporadic stress for young people in both Western and Asian Countries and has significant association with problems such as depression, anxiety, alienation and suicidal ideation.

Academic stress involves mental distress regarding anticipated academic challenges or failure or even an awareness' of the possibility of academic failure during school years, academic stressors can be seen in various

aspects of child's environment like home, neighborhood, school or friendship. It has been seen that people who are high on the emotional intelligence dimension are more likely to experience less negative impact by anxiety driven events. They have very good understanding of physical, mental and social consequences of negative emotions on the wellbeing and overall development and their relationship of life (Bhatt and Farooq, 2017). Further, Resilience is the ability to bounce back after disappointments or setbacks. It is the process and outcome of successfully adapting to difficulties of challenging life experiences, especially highly stressful or traumatic events and involves not only resisting failure under extreme circumstances but also positively recovering from those experiences. Therefore, resiliency enables students to find out solutions to imperfections that promote learned optimism, high self-esteem (Bhatt and Farooq, 2017).

Furthermore, educational deficit approaches are ignoring and downplaying interactions of wider structural factors that lead students to experience academic alienation. Thus, in modern educational setting, feeling of powerlessness and aloofness lead students to high dropout rate and high academic anxiety. This aloofness involves a rejection, stress, anxiety, alienation from dominant authorities, rules, and learning environment. Which creates a conflict between students and staff and students diverge to such a degree that it impacts negatively on their minds and adolescents become academic alienated. Consequently, the outcomes of alienated behavior are so serious and harmful that it not obstructs the growth of the education system but damage the personality of the individual. Definitely due these consequences and scarcity of research in this area especially in India provides a convincing rational to undertake further investigation into examining the relationship between academic alienation among adolescents in relation to emotional intelligence and resilience. Therefore, Investigator made an attempt to Academic alienation among adolescents in relation to emotional intelligence and resilience.

Objectives of the study

1. To find out the relationship between academic alienation and emotional intelligence among adolescents
2. To investigate the significance of relationship academic alienation and resilience among adolescents
3. To study the conjoint effect of emotional intelligence and resilience on academic alienation
4. among adolescents

Hypotheses of the study

1. There exists a significant relationship between academic alienation and emotional intelligence among adolescents.
2. There exists a significant relationship between academic alienation and resilience among adolescents.
3. The conjoint effect of emotional intelligence and resilience on academic alienation among adolescents is higher than their individual effects.

MATERIAL AND METHODS

The Present study was a descriptive survey method conducted on 500 adolescents studying in government and self-financed schools in the state of Punjab. The sample was drawn from ten randomly selected districts of Punjab. Multistage randomization was followed at the district, school and adolescent level. Data collection instruments are Academic anxiety scale for children (AASC) (2018) by Singh and Gupta, Mangal Emotional Intelligence Inventory (2018) EII-MM by Mangal and Mangal, The Resilience Scale (2009) by Wagnild and Young and Academic Alienation scale (2015) by Rita Rani.

Table 1. Showing coefficient of correlation between academic alienation and emotional intelligence among adolescents

Variables	Category	N	Correlation	Inference
Academic alienation and emotional intelligence	Adolescents	500	-0.12**	Significant

RESULTS AND DISCUSSION

In order to verify aforesaid hypothesis, coefficient of correlation was calculated with product moment method between the scores of academic alienation and emotional intelligence of adolescents. The coefficient of correlation between academic alienation and emotional intelligence of adolescents as depicted in Table No 1 is -0.12 which is significant at 0.01 level of confidence indicating that there is negative and significant relationship between the variables, show that more academic alienated adolescents are found to be less emotional intelligent and less emotionally stable too. This implies that higher the level emotional intelligence of adolescents, lower the academic alienation. Hence, the above stated hypothesis i.e. there exists a significant relationship between academic alienation and emotional intelligence among adolescents accepted. The finding is similar to the Shrivastava and Mukhopadhyay (2009); Kaur and Singh (2015); Mahmoudi, Brown and Saribagloo (2018) and Morinaj, Hadjar and Hascher (2020) who found that emotional intelligence is negatively correlated with

alienation, thus it indicates that individual with high emotional intelligence will be less alienated.

It can be seen from Table 2 that value of coefficient of correlation between the scores of adolescents on the variables of academic alienation and resilience is -0.23 which is significant at 0.01 level of significance. The value of correlation is significant and negative meaning thereby that, the adolescents who are more academically alienated are less resilient. Therefore, on the basis of above result, aforesaid hypothesis i.e. there is a significant between academic alienation and resilience among adolescents' is accepted. This finding is similar to the findings of Sakiz and Aftab (2019); Seligman and Csikzentmihaly, (2000) who found academic alienation has negative correlation with resilience. Li (2017) also advocated that resilient students are less academic alienated and more able to better cope up with academic stress. The study also reaffirms the importance of emotional intelligence and resilience as an important and indispensable part of adolescent's life which assists students to be industrious, disciplined, persistent, well equipped with coping strategies beyond the age and gender, more creative, better problem-solving skills, more strategic in making plans for the understanding of their own emotions and the emotions of others. Briefly speaking Emotional intelligence can prevent maladjustment and perceived stress through enhancing resilience in the academic context (Romano et al., 2019).

Table 2. Showing coefficient of correlation between academic alienation and resilience among adolescents

Variables	Category	N	Correlation	Inference
Academic alienation and resilience	Adolescents	500	-0.23**	Significant

Table 3. Showing conjoint effect of emotional intelligence and resilience on academic alienation among adolescents (N= 500).

Variable	R	R2	% Variance	F	Inference	Step-up Regression Equation
YX_1	0.120	0.014	1.4	7.26	Sig at 0.01 level	$Y=153.05-0.27X_1$
YX_2	0.233	0.054	5.4	28.54	Sig at 0.01 level	$Y=184.65-0.33X_2$
YX_1X_2	0.267	0.071	7.1	19.08	Sig at 0.01 level	$Y=217.06-0.30X1-0.34X_2$

Regression for predictive efficiency: To test this hypothesis, the step-up regression technique was employed. The square of multiple correlation (R^2), called the coefficient of correlation The conjoint effect of emotional intelligence and resilience on academic alienation among adolescents is higher than their individual effects.

Y - Academic alienation, X1 - Emotional intelligence, X2 - Resilience

The effect of emotional intelligence on Academic alienation among adolescents was found significant at .01 level (F (1, 498) =7.26). The computed value of R2 of emotional intelligence and Academic alienation among adolescents (YX1) is 0.014 which indicates that the contribution of emotional intelligence on Academic alienation among adolescents is 1.4%. The Academic alienation among adolescents can be predicted with the equation

Academic alienation=153.05-0.27x Emotional intelligence

i.e. for every unit of increase in emotional intelligence, Academic alienation among adolescents decrease. 27 The effect of resilience on Academic alienation among adolescents was found significant at .01 level (F (1,498) =28.54). The computed value of R2 of resilience and Academic alienation among adolescents (YX1) is 0.054 which indicates that the contribution of resilience on Academic alienation among adolescents is 5.4%. The Academic alienation among adolescents can be predicted with the equation

Academic alienation= 184.65-0.33 x Resilience

i.e. for every unit of increase in resilience, Academic alienation among adolescents decrease .33

The conjoint effect of both emotional intelligence and resilience on Academic alienation among adolescents was found significant at 0.01 level of significance (F (2,517) =19.08). The computed value of R2 of Academic alienation with emotional intelligence and resilience (Y1X1X2) is 0.071 which indicates the contribution of emotional intelligence and resilience on Academic

alienation among adolescents is 7.1%. As %age variance(=7.1) of variables of emotional intelligence and resilience conjointly on Academic alienation among adolescents shows increase in its value from emotional intelligence (%age variance=1.4) and resilience (%age variance=5.4), it indicates that the conjoint effect of emotional intelligence and resilience on academic alienation among adolescents is higher than that of emotional intelligence and resilience separately.The achievement in social studies among male adolescents can be predicted with the equation.

Academic alienation=217.06-0.30 x emotional intelligence -0.34 x resilience. Hence, hypothesis 3 stating, "The conjoint effect of emotional intelligence and resilience on academic alienation among adolescents is higher than their individual effects" stands accepted.

CONCLUSION

The findings of the present research divulge that emotional intelligence and resilience are important factors in reducing the level of academic alienation. The results reveal that there is a significant and negative relationship between academic alienation and emotional intelligence among adolescents. A significant and negative relationship was also found between academic alienation and resilience among adolescents. More academically alienated adolescents are found to be less emotionally intelligent, less emotionally stable and less resilient too. It is quite apparent from the regression model summary that emotional intelligence and resilience would contribute towards the prediction of academic alienation of adolescents both independently as well as conjointly. Present study also explored that there is a negative relationship between academic alienation, emotional intelligence and resilience. The result suggests that more resilient and emotionally intelligent adolescents have lower perception of stress and academic alienation. Resilient students have an extraordinary coping ability. They keep trying and 'bouncing back' despite adverse, challenging or threatening circumstances.

These emotionally intelligent and resilient adolescents tend to have 'the heightened likelihood of success in school and other life accomplishments. The protective factors that have an effect on the resistance of the person against risk factors are composed of internal and external factors. Internal control focus and self-control, empathy, active problem-solving skills, positive personality traits, realistic plans by taking appropriate steps to realize them, effective management of emotions, sense of humor, optimistic viewpoint, intelligence, self-confidence and self-possessing value are individual protective factor. Thus, emotional intelligence and resilience are considered as inherent strengths which equip the adolescents to deal with Academic Alienation by enhancing their ability to cope with the academic stress and anxiety. Thus, there is a need to study the relationship between academic alienation, emotional intelligence and resilience. Hence, it is obvious from the results stated earlier that the emotional intelligence and resilience are the most significant and influential contributor in predicting academic alienation among adolescents.

Academic Alienation is not simply a phenomenon rather it is a kind of disorder which has far reaching negative impact at personal and social level and sad part of the story is that it is becoming a prominent feature among our younger generation. The study which worked on the emotional intelligence and resilience among adolescents on their academic alienation will in turn be benefitted to planners, educational authorities, teachers and parents to understand academic alienation in right perspective and help them in providing suitable environment at home and schools for enhancing their emotional intelligence and resilience. As the results of the study indicate that emotionally intelligent and resilient student has less academic alienation, it will help teachers, parents and society at large understand the need for co-operation and collaboration within the classroom to develop positive relationships and encourage students to listen and understand to others. Parents and teachers should work on making the students accept and fight with adversities and should believe in themselves instead of succumbing to those failures

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Antioxidant and Antibacterial Activities of Flower and Fruit of *Sterculia alata*

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ABSTRACT

Sterculia alata belongs to family Sterculiaceae. It is a highly medicinal plant and its uses are already in practice in traditional medicines. Its leaves and bark have shown various biological activities. But there is utmost need to identify medicinal potential of other parts of the plant. Extracts of fruits and flowers were prepared in different solvents. Antioxidant activities were assessed by using two most common methods- through β-carotene and Linoleic acid assay and Hydrogen Peroxide (H_2O_2) scavenging assay. Disc diffusion methods were used to study antibacterial activities of extracts. Both fruit and flower extracts showed antioxidant and antibacterial activities. Aqueous fruit extract showed maximum hydrogen peroxide scavenging activity (80.13±0.29), whereas methanolic extract of flower extract showed comparatively less hydrogen peroxide scavenging activity (78.25±0.12). Highest antioxidant activity (77.78±0.58) was observed by aqueous extract of fruit through β carotene/ linoleic acid assay. Lower antioxidant activity was shown by flower extract (ethanolic extract: 52.25±0.29). Antibacterial efficacies of fruit and flower extracts were observed against six resistant pathogenic bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis* ATCC, *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*). Flower extract showed maximum inhibition for *E. coli* (aqueous extract: 13.00± 0.29) and *K. pneumoniae* (methanolic extract: 13.33±0.67). Fruit extracts were found effective against *S. aureus* (aqueous extract: 13.00± 0.58), *E. faecalis* (methanolic extract: 15.00±0.04), *P. mirabilis* (methanolic extract: 14.00±0.29) and *P. aeruginosa* (ethanolic extract: 10.67±0.33). Both fruit and flower extracts can serve as the source of natural antioxidant and antibacterial.

KEY WORDS: PLANT EXTRACT, ANTIOXIDANT, ANTIBACTERIAL, INHIBITION ZONE.

INTRODUCTION

Medicinal plants and their parts have been identified as highly useful in treating various diseases since ancient time. Several phytochemicals have shown their efficacies in treating many chronic and deadly diseases such as cancer, diabetes, neurological disorders, AIDS, etc. World Health Organization (WHO) has also witnessed the dependence of about 80% of world population over plant products for medicines. Pharmaceutical activities of any plant is directly associated with the types and concentration of its bioactive compounds (Shihabudeen et al, 2010: Gowri and Vasantha, 2010). Different solvents show different extraction efficiency and therefore, extracts prepared in different solvents show considerable different phytochemical activities (Ernst and Kuppan, 2013: Ngo et al, 2017).

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Sedentary life style, poor intake of healthy diet, mental stress and illness are some major reasons for oxidative stress. Oxidative stress causes formation of free radicals. Reactive oxygen and reactive nitrogen species (ROS and RNS) are two major groups of free radicals (Tiwari et al, 2009: Meo et al, 2016). ROS and RNS are being produced due to oxidative stress and cell metabolism. Antioxidants play pivotal role in scavenging of free radicals by inhibiting or delaying process of oxidation and thus they contribute greatly in detoxifying organisms. Nowadays, plant products have been proved as efficient antioxidants and due to their nil or negligible side effects, they are considered superior over synthetic antioxidants. Many plants also show antimicrobial activities due to their phytochemicals. For example, terpenes, phenolics, defensins, essential oil, etc present in plant show potential antimicrobial activities. Such plant products can be good alternative of antibiotics and can deal effectively with multi drug resistant problem (Laws et al, 2019; Pacios et al, 2020).

Sterculia alata Roxb. syn. *Pterygota alata* popularly known as Buddha Coconut, is a large fast growing moist, deciduous and evergreen bark tree. It belongs to Sterculiaceae family. It is distributed mainly in tropical Asia (Lin et al, 2010). In India, it is found in western ghats, south and central Sahyadris. The plant is an important forest tree and medicinally very important. Due to fast growth, its plantation is being done for afforestation purpose. Presence of several phytochemicals (phenolics, flavonoids, steroids, anthraquinones) have been identified from leaf and stem extracts of *Sterculia alata* (Jahan et al, 2014; Jahan et al, 2014; El-Sherei et al., 2018). Omran et al (2019) have observed antioxidant and antimicrobial activities of leaves of *Sterculia alata*. Various studies showed medicinal importance of *S. alata*. Biosynthesis and accumulation of secondary metabolites in different organs of same plant may be different and it has been observed by their varied distribution patterns at tissue and organ level (Zribi et al, 2014; Azadeh et al, 2020). Thus, extensive study is needed to identify all possible phytochemicals of a plant and to categorize their biological activities. Flowers and fruits of Sterculia alata are still unexplored and identification of their biological activities is highly required. Present study deals with the study of antioxidant and antibacterial properties of flower and fruit extracts of *Sterculia alata*.

MATERIAL AND METHODS

Collection of Plant Material: Flowers and fruits of S. alata were collected from the campus of Banaras Hindu University, Varanasi during the month of February and March. Flowers and fruits were cleaned under running tap water. Pericarp (separated from fruit) and flowers was shade dried, oven dried at 40-45°C for 2 hour and then grinded in mechanical grinder to make course powder. Extraction was done from 20g of flower and fruit powder in 250 mL of solvent by using a soxhlet apparatus for 24 hours. Ethanol, methanol and double distilled water were used as solvents for extraction. Extracts were then dried at 40°C in rotary evaporator. Extracts were stored at -20°C till use. Percentage yield (w/w) of crude extract was calculated using formula:

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

Where PY is percentage yield of extact

Preparation of Sample Extract for Different Assays: To prepare stock samples, about 100 mg extract was dissolved in 50 ml of respective solvent, final concentration 2mg/ml for antioxidant assays. Different volume of samples was used from the stock for various experiments. Stock samples of concentration 100 mg/ml in dimethyl sulphoxide (DMSO) was prepared for antibacterial activity. For the test of susceptibility about 5µl of extracts was taken onto sterile disc.

Antioxidant Activity through β-carotene and Linoleic Acid Assay: Elzaawely et al (2007) method was used with some modifications for β-carotene bleaching assay. For the preparation of stock solution, β- carotene of concentration 2mg/ml was dissolved in chloroform and mixed with 20 µl of linoleic acid followed by addition of 200µl of Tween-20 in a round bottom flask. 50 ml of double distilled water was added in the residue left after complete evaporation of chloroform with vigorous stirring to form an emulsion. Extract (800 µl) was added in the test tube containing 2400µl of emulsion and immediate absorbance was recorded at 470 nm against the blank solution. After that tubes were incubated for 2 h at 50o C then absorbance was recorded. For control, DMSO was added in emulsion in place of plant extract. Percent inhibition was calculated as

$$I\% = \frac{\text{Absorbance of } \beta - \text{carotene after 2 h}}{\text{absorbance of } \beta - \text{carotene initial}} \times 100$$

Hydrogen Peroxide (H_2O_2) Scavenging Assay: Hydrogen peroxide scavenging activity was assessed according to Bokhari et al (2013) with some modifications. For stock solution, H_2O_2 (4mM) solution was prepared in phosphate buffer (50 mM, pH 7.4). 2 ml (100mg/ml) of plant extract was mixed with 3 ml of H_2O_2 and absorbance at 230 nm was recorded after 10 min. Phosphate buffer without H_2O_2 was served as blank while H_2O_2 solution without sample is taken as control. Percent hydrogen peroxide scavenging activity was determined as

$$I\% = \frac{\text{Absorbance of control} - \text{Absorbance of samples}}{\text{absorbance of control}} \times 100$$

Media Preparation and Test Microorganisms for Antibacterial Assay: For bacterial media preparation, Muller Hinton agar (38g/L) and agar (10g/L) were dissolved in double distilled water. Saline (8.5 g/L) was prepared by dissolving in double distilled and autoclaved for 15 min at 1.1kg/cm2 and 121°C. About 20 mL sterile

media was used for plating. Bacterial cells were grown on MHA (Himedia, Mumbai) for 24 h at 37 °C to form bacterial inoculums. The bacterial suspension turbidity was maintained at 0.5 McFarland turbidity standards (approximately 1x107 CFU/mL). For assessment of antibacterial activity, six bacteria (both gram positive and gram negative) were selected. Test organisms selected were *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis* ATCC, *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*. Bacterial cultures were obtained from Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India.

Broth cultures of young bacteria were maintained for screening experiments. For antibacterial activity, disc diffusion method (Murray et al., 1995) was used. Bacterial suspension was evenly distributed over the surface of solidified media by using a sterile swab and allowed to dry for 5 min. About 5µL extract was loaded to each sterile disc followed by placing disc on medium surface. DMSO was taken as negative control and plates were incubated in BOD (Remi) incubator for 24 h at 37 °C. Specific standard drug Streptomycin was used against all Gram positive and Gram negative bacteria. Zones of inhibition were measured in millimeters.

Statistical analysis: All experiments were carried out in triplicates and repeated thrice independently. Data were presented by using SPSS software (version 16 ,Chikago, USA). Data were represented as mean ±SE.

RESULTS AND DISCUSSION

Oxidative stress induced by free radicals is the root cause of several diseases. Intake of antioxidants– both synthetic and natural provides protection from the damage of free radicals. They act as free radical scavengers and prevent or delay the process of oxidation. Plants are most commonly known reservoir of natural antioxidants and they can serve as the source of cost-effective antioxidants. Natural antioxidants generally show almost negligible side effects. Several secondary metabolites such as phenolics, flavonoids, carotenoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, etc. show antioxidant activities (Hollman, 2001; Sasikumar and Kalaisezhiyen, 2014). Similarly, many phytochemicals such as phenolics, flavonoids, alkaloids, essential oils, etc have shown strong antimicrobial activities (Silva and Fernandes, 2010; Seyidoglu and Aydin, 2020).

β- carotene and linoleic acid assay is one of the rapid method to screen antioxidants. Two or more methods are preferred for the assessment of antioxidant activities of any plant specimen to get more reliable result. Therefore, another method to analyze antioxidant activities i.e. H_2O_2 scavenging activity was also performed. H_2O_2 scavenging activity of extracts may be due to their antioxidant constituents present in them which donate electrons to H_2O_2 to neutralize it into H2O (Ebrahimzadeh et al., 2009).

In present study, antioxidant activities of fruit extracts in all solvents were observed better than flower extracts. Antioxidant potential of fruit was observed maximum in aqueous extract (77.78±0.58), whereas activities in methanolic (67.56±0.54) and ethanolic (60.91±0.45) extracts were also significantly high (Fig 1). In flower, maximum antioxidant activity was recorded in ethanolic extract (52.25±0.29), then in aqueous extract (27.84±0.28) and minimum in methanolic extract (11.88±0.32) (Fig 1). Hydrogen peroxide scavenging activity was also observed higher in fruit extract than flower extract. In fruit extract, maximum scavenging activity was reported in aqueous extract (80.13±0.29), significant activity was observed in methanolic extract (62.88±0.08) and minimum in ethanolic extract (28.31±0.69). In flower, methanolic (78.00±0.12) and aqueous extracts (77.14±0.10) showed significant responses (78.00±0.12), but ethanolic extract (10.11±0.31) was not observed effective.

In present work, six antibiotic resistant test microorganisms were taken to assess antimicrobial effect of plant extracts. All test organisms selected were resistant (Cheesan et al, 2017) and their details are as such- *S. aureus* (Penicillin resistant; Methicillin resistant *S. aureus*-MRSA), *Enterococcus faecalis* (vanomycin resistant), *Klebisiella pneumonia* (XDR-extensively drug resistant), *Proteus mirabilis* (chloramphenicol and erythromycin resistant) and *E.coli* (MDR -multi drug resistance), *Pseudomonas aeruginosa* (Zerbaxa resistant). Antibacterial activities of plant extracts will pave novel way of treatment of diseases caused by them. The current number of antibacterials is not enough for controlling the evolution of MDR and XDR bacteria. Therefore, many pharmaceutical companies are in search of new antibacterial which can reverse the mechanism of bacterial resistance (Isah, 2019; Pacios et al, 2020).

Flower and fruit extracts were taken for the assessment of their antibacterial activity. Results of antibacterial activity are shown in Table 1 and 2. Flower and fruit extracts exhibit antibacterial activity against most of the pathogenic bacteria with different potency. Flower extract showed significant inhibition against *E. coli* (13.00±0.29), *E. faecalis* (12.33±0.17), *K. pneumonia* (13.33±0.67) and *P. mirabilis* (13.83±0.88), but was found least effective against *S. aureus* (maximum in aqueous extract- 10.33±0.67) and *P. aeruginosa* (maximum in ethanolic extract- 8.67±0.67). Fruit extract showed significant antibacterial potential against *E. faecalis* (15.00±0.04), *P. mirabilis* (14.00±0.29), *K.pneumoniae* (13.30±0.17), *S.aureus* (13.00±0.58) and *E.coli* (11.67±0.44), but inhibition was comparatively low against *P. aeruginosa* (10.67±0.33).

Table 1. Antibacterial activity of flower extract of *Sterculia alata*

Test organisms	Inhibition zone diameter (mm)				
	Aqueous	Ethanolic	Methanolic	Control	Standard (5µg/ml)
E.coli	13.00±0.29	11.83±0.58	9.17±0.60	0.00±0.00	34.17±0.16
S.aureus	10.33±0.67	7.17±0.60	10.00±0.58	0.00±0.00	32.33±0.33
E. faecalis	10.33±0.33	12.33±0.17	10.50±0.29	0.00±0.00	41.50±0.76
K. pneumonia	7.33±0.89	10.00±0.58	13.33±0.67	0.00±0.00	35.50±0.29
P. mirabilis	8.67±0.33	13.83±0.88	12.83±0.83	0.00±0.00	17.33±0.89
P.aeruginosa	6.00±0.58	8.67±0.67	7.67±0.17	0.00±0.00	42.33±0.17

Data are means of three replicates (n=3) ± standard error.

Table 2. Antibacterial activity in fruit extracts of *Sterculia alata*

Test organisms	Inhibition zone diameter (mm)				
	Aqueous	Ethanolic	Methanolic	Control	Standard (5µg/ml)
E.coli	11.67±0.44	11.00± 1.15	7.83±0.44	0.00±0.00	39.00±0.50
S.aureus	13.00±0.58	10.30±0.17	12.50±0.29	0.00±0.00	37.50±0.29
E. faecalis	6.17±0.60	9.66±0.33	15.00±0.04	0.00±0.00	35.17±0.60
K.pneumoniae	0.00±0.00	9.50±0.29	13.30±0.17	0.00±0.00	42.33±0.17
P. mirabilis	0.00±0.00	11.00±0.50	14.00±0.29	0.00±0.00	18.00±0.58
P.aeruginosa	6.80±0.44	10.67±0.33	8.50±0.76	0.00±0.00	45.17±0.13

Data are means of three replicates (n=3) ± standard error.

Figure 1: Antioxidant activity through β-carotene/linoleic acid bleaching assay in flower and fruit extract of *Sterculia alata*

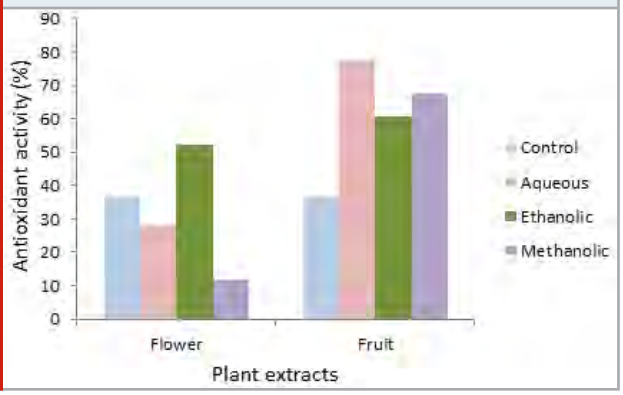
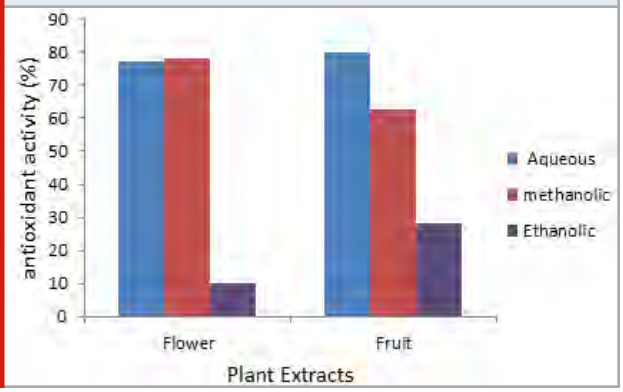


Figure 2: Hydrogen peroxide scavenging activity in the flower and fruit extracts of *Sterculia alata*.



There are many factors, which affects antibacterial potential- type of extract used, its concentration, solvents used in extraction and type of bacteria. Extracts prepared in different solvents show different antioxidant and antimicrobial activities due to different dissolving capacities of metabolites (Altemimi et al., 2017 and Ngo et al., 2017). There are many mechanisms by which phytochemicals act against microbes- by disrupting cell wall of bacteria (Burt 2004, Gill and Holley 2006) or by changing the permeability of bacterial cell membrane and mitochondria (Tiwari et al., 2009). Resistance gene of the bacteria may code for efflux pumps which eject antibiotic from the cells or by inducing enzymes

for degradation or inactivation of antibiotic. Many plants possess MDR pump inhibitors to enhance the activity of their own natural antimicrobial compounds. Extracts of such plants can be highly effective if used in combination of ineffective or resistance prone antibiotics (Morel et al, 2003; Abreu et al, 2012). Thus, plants rich in secondary metabolites show significant antioxidant and antimicrobial properties and it is utmost need to explore such plants (Isah, 2019; Pacios et al, 2020).

CONCLUSIONS

Sterculia alata is an important medicinal plant and all parts of the plant have shown its high pharmaceutical importance. Both fruit and flower extracts showed significant antioxidant activities thus paving way for the synthesis of cost effective antioxidants. Antibacterial activities against resistant bacterial strains show its use in future as alternative antibacterial. In future, its extracts may be used for combinational therapy to combat bacterial resistance against antibiotics.

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Inhibitory Effects of Acaciasides Isolated from the Funicles of *Acacia auriculiformis* on the growth of *Escherichia coli*

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ABSTRACT

The present study was carried out to establish the effect of acaciaside on Gram positive and Gram negative bacterial community especially to *Escherichia coli*. The inhibitory effect of acaciaside on growth of typical intestinal gram negative pathogen *E.coli* was identified. The degree of inhibition was measured by well disc assay method. In recent days, antimicrobial resistance has become a great global threat to public health systems worldwide. Bacteria pose the greatest threat to human health because of its growing resistance to antibiotics are the members of the enterobacteriaceae family, mainly *E.coli*. *E.coli* is an important contaminant of drinking, agricultural, industrial and recreational water which is a major environmental and public health concern. Acaciaside A and acaciaside B were isolated individually from the funicles of *Acacia auriculiformis*. The mixture of these two acylated triterpenoid bisglycoside saponins are known to have antihelminthic and antimicrobial activity. Here antibacterial activity of the individual compound has been investigated.

Due to continuously increasing number of infections caused by multidrug-resistance *E.coli* as they are transmitted through fecal-oral route among humans and from other environmental sources, the better understanding of the epidemiology of this strain and their mechanism of resistance are key components to cure against their infections. Acaciaside A inhibited the growth of *Escherichia coli, Salmonella typhimurium* and *Bacillus megaterium* at 200, 400 and 600 µg/ml, respectively whereas acaciaside B inhibited the growth of *Pseudomonas aeruginosa* at 600 µg/ml. The present investigation reveals the inhibitory effect produced by acaciaside A or in combination with acaciaside B in *E.coli*, and in comparison with other bacterial strain. By this inhibitory effect of acaciaside which acts as a natural product we can minimize the growth of several species of harmful bacteria. In conclusion, *E.coli* revealed a great deal for its presence in the environment, its diversity as well as its main role in the human microbiome and disease. This findings also outcomes its biology and ecology for better understanding of its growth inhibition.

KEY WORDS: ACACIASIDE A AND B, ANTIHELMINTHIC, ANTIMICROBIAL, INHIBITORY EFFECT, SAPONINS.

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INTRODUCTION

During ancient times, plants are the important source of natural products for maintaining normal human life; they are used as natural therapies. According to World Health Organization (Santos et al.1995) all medicinal plants around the world are of good source to obtain a variety of important drugs. *Acacia auriculiformis* is one of the important therapeutic plant from which many valuable compounds have been isolated. The active principle, which was isolated from the funicles of *A. auriculiformis* contains two triterpenoid saponins, acaciaside A and acaciaside B which have antifilarial activity against *Setaria cervi*. Its native origin is in Australia but came in India in 1946 in West Bengal (Kushalapa 1991). It is also important for making paper, furniture's etc. and has medicinal and forestry importance (Singh et al.2007). The growth of *A. auriculiformis* is very fast generally first year of its development, its growth rate is also high 2-3 m per year even in low fertility soil (Pinyopusarerk 1990) By adding nitrogen to soil and mixed with other species *A. auriculiformis* provides enhanced productivity in early thinning operations (CABI 2013).

Acacia auriculiformis is also known as ear leaf *Acacia* which is an important medicinal plant and widely distributed group of fabaceae (Gijasahnkar 2010). This plant acts as safe and effective substitutes for chemical control against several types of pathogens (Ouassat et al.2020). Many parts of the plant used in traditional medicine such as bark is used as a remedy from rheumatism in Australia, its seeds and roots are used in the treatment of skin diseases and sore eyes respectively (Singh et al. 2007). The plant is also useful as antimalarial drug in many parts of the world as in Nigeria (Okokon et al. 2010). It acts as an antifilarial (Ghosh et al. 1993, Mahato 1996), antioxidant (okokon et al. 2010), antidiabetic (Sathya et al. 2013), antimutagenic (Kaur et al. 2002) compound. Ethylacatate fraction of *A. auriculiformis* was found to be most potent extract which have protein kinase inhibitory activity and another ethylacetate fraction, 3, 4', 7, 8-tetrahydroxyflavone was isolated and it is also a potent inhibitor of DYRK1A and CDK9 which proves the plants anticancer and intiflammatory property (Ahmadu et al. 2019).

Acaciaside generally grows in humid areas and also rich in glucuronic acid, methylglucuronic acid, arabinose, rhamnose and galactose (Anderson 1978). Their antibacterial and antifungal activity was proved in several bacterial and fungal strains. In *Aspergillus ochraceous* and *Curvularia lunata* complete inhibition of conidial germination was recorded at 300 µg/ml or less but in case of *Salmonella typhimurium, Pseudomonas aeruginosa* and *Bacillus megaterium* 700 µg/ml or higher concentrations of the mixture was required for inhibition of their growth (Mandal et al. 2005). *E.coli* the gram negative bacteria which normally resides as intestinal flora in humans, and also acts as an indicator species of fecal contamination to assess the safety and quality of water (US EPA 1986).

Although *E. coli* maintains a friendly environment for its anaerobic neighbors by consuming oxygen that enters the gut (Chang et al. 2004), most of them are harmless to humans but certain strains are pathogenic and causes several types of fatal diseases such as bloody diarrhea, watery diarrhea, meningitis, urinary tract infection, and sepsis, which can lead to human death (Nataro et al. 1998, Gyles et al. 2007). *E.coli* also causes worldwide infections neonatal meningitis, bacteremia and also traveler's diarrhea (Peleg et al. 2010).Urinary tract infections is the most common in many countries causing community and hospital acquired urinary tract infections (gajdacs et al. 2019). A recent WHO report mentioned that medicinal plants around the world are one of the good sources of new important drugs (Efferth 2017). There are several examples of these compounds isolated from many plants that have been proved to be effective as antimicrobial agents.

In this research, antibacterial activity of acaciaside A and acaciaside B or their mixture were performed to investigate the inhibitory effect of the samples obtained from *Acacia* plant on the activity of drug-resistant *E.coli*. This research plays a foundation for the further development of antibacterial agents and also identify the least MIC (minimum inhibitory concentration) value for *E.coli* in comparison to other bacteria for sustainable normal human life.

MATERIAL AND METHODS

Plant material: *Acacia auriculiformis* A.Cunn (Mimosaceae) funicles collected from Santiniketan was authenticated by Department of Botany, Visva-Bharati University. The dried and powered leaf extracts of *Acacia auriculiformis* mixing up with 70% ethanol for 72 hours. Then it is vaccum at 38^{0}C and the extract was stored in a refrigerator at 4^{0}C for future use.

Use as traditional medicine: Different extracts of the plant material are used against filaridis and helminthes (Ghosh et al. 1993, Ghosh et al. 1996). It has also been examined for its spermicidal activity (Pakrashi et al. 1991) and also for fungicidal activities against *Aspergillus ochraceous* and *Curvularia lunata* (Mandal et al. 2005).

Experimental material: Acaciaside A, Acaciaside B and mixture of acaciaside A and acaciaside B obtained from *Acacia auriculiformis* was used in this experiment (Ghosh et al.1993).

Studied activity: Experiment was conducted to study antibacterial activity after treating with sublithal concentration of acaciaside A, acaciaside B and the mixture of acaciaside A and acaciaside B (Mandal et al. 2005).

Used microorganisms in the experiment: Four bacterial strains belonging to both Gram positive and Gram negative categories (Table 1) were procured from Microbial type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

RESULTS AND DISCUSSION

Antimicrobial activity of acaciaside A and acaciaside B and the mixture of acaciaside A and acaciaside B against four bacteria are reported in (Table 1).

Acaciaside A and acaciaside B singly or in mixture produced inhibitory effect on the growth of bacteria tested. However, the minimum inhibitory concentration of the two saponins when tested against bacterial strains appears to differ. Acaciaside A completely inhibited *E.coli* at 200 µg/ml as did the mixture of two saponins, whereas acaciaside B inhibited completely at 600 µg/ml. Similar results were obtained in *S.typhimurium* and *B.megaterium* where acaciaside A singly or in combination of acaciaside B contributed as the major inhibitory substance. It appears from the results that bacteriostatic activity of saponins may be mediated by acaciaside A. Acaciaside B completely inhibited *P. aeruginosa* at 600 µg/ml as did the mixture of two saponins, which indicate that the bacterioststic effect of saponins on *P.aeruginosa* may rest with acaciaside B. Previuosly it was reported that the mixture of two saponins inhibited the growth of *P.aeruginosa* and *S.typhimurium* at 700 µg/ml (Mandal et al. 2005).

Table 1. MIC (minimum inhibitory concentration) value of acaciaside A and acaciaside B against different bacterial strains

Test Compound (µg/ml)	*E.coli* MTCC 68 Gram Negative	*P.aeruginosa* MTCC 741 Gram Negative	*S.typhimurium* MTCC 98 Gram Negative	*B.megaterium* MTCC 1684 Gram Positive
Acaciaside A	200	More than 1000	400	600
Acaciaside B	600	600	600	1000
Acaciaside A+B	200	600	400	600

Here it is suggested that saponins-induced bacteriostatic activity may be strain specific i.e. either Gram +ve or Gram –ve bacteria. In case of *E.coli* it was noticed that their minimum inhibitory treatment dose either by acaciaside A or their combinationis is only 200.When acaciaside A and B applied in combination or acaciaside A singly to the growing culture, they produced almost similar kind of effects on the inhibition of *B. megaterium* cells. Major deviation occurs due to stress induced by the compounds at early phases of sporogenesis process. Change of morphology of different Gram positive and Gram negative bacteria were reported due to change in nutritional conditions or stress in the growth environment an even in the growth phase dependent process (Rasanen 2002, Sawer et al. 2005).

Kumar et al, showed that the ethanolic, ethyl acetate, and water extracts with *Acaciaside* were found be active against certain bacteria such as *E.coli* and fungi. Many evidences till date shows that the extracts obtained from medicinal plants are effective as antimicrobial agents. But no reports have claimed to observe that bacteria developing resistance to plant-based antimicrobials (PBAs) (Cheesman et al. 2017). In the present investigation inhibitory effect produced by acaciaside A, or in combination with acaciaside B in some bacterial cells was clearly established. As inhibition of *P.aeruginosa* is not affected very much by saponins probably a different mechanism is operating in the process of inhibition. But in case of *E.coli* minimum inhibitory concentration was noticeable perfectly.

CONCLUSION

E.coli has a variety of strains that ranging from commensal residents of the gastrointestinal tract to mixed pathogens that are able to create several illnesses. The present investigation aims to discuss the least minimum inhibitory concentration *Acacia auriculiformis* on bacterial population particularly *E.coli*. This plant has many medicinally important phytoconstituents which prove their pharmacological activities. Inhibition of *E.coli* growth by acaciaside A or their combination insights a significant outcome and better understanding in medicinal purposes for future human life.

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Cellulase Production by Fungi from Agro Wastes under Solid State Fermentation

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ABSTRACT

Microbial cellulases find applications in many industries and constitute a significant share of the world's industrial enzyme market. In order to improve the cost function of the cellulase producing processes with enhanced yield and novel activities, superior bioprocesses are formulated these days. The current study was designed to isolate and identify superior cellulose-degrading fungi from cellulosic waste and selection of different cellulosic waste like paper waste, cotton ginning, wheat Bran and sugarcane bagasse for cellulase production under solid state fermentation. Various physical parameters such as temperature, pH and incubation time were optimized for induced cellulase production from *Trichoderma sp*. in SSF. Among 14 fungal isolates, four isolates were selected by primary screening of CMCase method. Solid state fermentation was carried out with four Agro wastes such as waste paper, cotton ginning waste wheat bran and sugarcane bagasse. Among 4 fungal strains, A11 isolated which was identified as Trichoderma sp. were selected by secondary screening showing higher cellulase activity on solid state fermentation and wheat bran was found to be the best substrate for cellulase production. In the present study the maximum enzyme production by fungal isolate A11 using Wheat bran as substrate showed 8.49 U/g and 2.23 U/g activities of CMCase and FPase respectively recorded at 35°C on 120 hrs of incubation period at 5 pH. Isolation of cellulase producing fungal strain will help in bio-conversion of cellulosic materials to glucose and other fermentable sugars which can be further used for the production of bio-ethanol.

KEY WORDS: AGRO WASTES, CMCASE, FPASE, SOLID STATE FERMENTATION.

INTRODUCTION

All over the world, there is a growing concern about the over dependence on fossil fuels as well as their possible role in global warming. Because of this, there is a huge search for a biofuel to use as an alternative source of energy, utilizing the existing lignocellulosic biomass as a substrate for bioethanol production. (Cai et al., 2019). Exploiting the full chemical potential of cellulosic waste for the energy industry can be sustainably mediated by enzymes called cellulase. Till date fungi are the main cellulase producing microorganisms, though a few filamentous fungi of Trichoderma sp. and Aspergillus sp. are the main source for cellulase production (Xue et al., 2017; Ezeilo et al., 2017). This group of multi-enzyme complex of the glycosyl hydrolase family consist of mainly endo-b-1,4-glucanase, exo-b-1,4- glucanase (cellobiohydrolase) and b-glucosidase. The complete hydrolysis of cellulose to fermentable sugar showing that the process is completed by the cellulase enzymes synergistically breakdown the homogenous polysaccharide (Srivastava et al., 2018; Waill et al., 2019). Although traditionally, the enzymes of industrial importance have traditionally been produced in submerged fermentation

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(SmF) because of the ease of handling and good control of environmental factors such as temperature, aeration, agitation and pH (Singh et al., 2007). However, solid state fermentation (SSF) techniques have to be found to be better adapted to increase the yield and additionally due to the ability of filamentous fungi to grow well on solid substrates which reduces the cost of enzyme production. (Hui et al., 2010; Waill et al., 2019).

The use of low cost agri- cultural waste adds value to SSF process as it not only makes it cost effective but also promotes a substantial reduction in environmental pollution by agro-industrial residues A part of this it is has been reported that SSF is to be most appropriate process for developing countries due to other benefits such as maximum productivity, simple technique, low investment, energy requirement low and less water requirement, good product recovery and lack of foam build up (Zeng and Chen, 2009; Souza and Magalhaes, 2010; Ezeilo et al., 2020). Thus, Cellulases enzymes are allowed for application in various industries; pulp and paper industry, textile and bioethanol industries, wine and brewery industry, food industries, animal feed industry, agricultural and detergent industries (Darwesh et al. 2020).

The current study is concerned with the hyper-production of cellulase using low-cost agro-industrial residue as a substrate for fungal co-culture using SSF. Selection of agro waste as a carbon source and optimization of various physical parameters for production of cellulase from the co-culture of Trichoderma sp. which was isolated from wheat bran in solid state fermentation. Further, the use of whole fermented broth for saccharification of lignocellulose was compared with the free enzyme in order to minimize the unit operations intended for economic feasibility. The applicability of cellulase was analysed for saccharification of various lignocellulosic substrates.

MATERIAL AND METHODS

Isolation of Cellulose-Degrading Fungi: For isolation of culture samples were collected from paper industry waste, wheat bran waste, soil sample and wood furnishing were collected from different site of Gandhinagar and Kadi in sterile polythene bags. Isolation and primary screening of cellulolytic fungal species was done on Carboxyl agar medium (CMC). The cellulose degradation by fungal were tested using the media proposed by Hart et al. (2002).

Streak plate method: Streak plate method is best method for isolation of fungi. First streak is made with the mix colonies then other are continue of the previous strike using separate sterile tooth peck for isolation of pure culture from mix colonies.

Sprinkled method: Sprinkled method is useful for the soil samples and sawdust. Soil samples were sprinkled on the potato dextrose plate. Finally, petri dishes were incubated at 300C for fungal growth. Pure colonies were separated from mix colonies after 72 hr of incubation at 30^{0}C.

Serial dilution method: Serial dilution method is use to reduce dense culture to a usable concentration. 0.5 gm of samples were taken and add in 9 ml of sterile distill water and mix well with the help of vortex for 10 min. From this stock solution, 10-1 to 10-5 serial dilutions were prepared. 1 ml from dilution medium inoculated into Potato dextrose agar medium. The plats were incubated for 4 to 5 days at 300C for observation of the fungi.

Primary screening of cellulose degrading fungi: The individual microorganism was grown on basal salt media supplemented with 1% Carboxy methylcellulose (CMC) medium. The pure cultures were inoculated in the center with almost equal amounts and incubated at 30^{o}C until substantial growth was recorded. The petri plates were incubated at 50^{o}C for 30 min. Plates were flooded with 1% Iodine solution and allowed it to stand for 5-10 minutes. The clear zone was observed around the colony due to hydrolysis of cellulose by cellulase which is produced by fungi.

Characterization and Identification of fungal Isolates: Fungal colonies were isolated form different samples for cellulase producing microorganisms by serial dilution method. The isolates were inoculated on sterile PDA plates and incubated at 30°C for 48 hours in order to obtain a pure culture. The various isolates were determined based on Morphological characteristics and microscopic examinations and the reproductive and vegetative structures were also studied (Devanathan et al., 2007).

Culture Maintenance: Isolated fungi strain was maintained on Potato dextrose agar (PDA) slants. The pH of the medium was maintained as neutral and culture was incubated at 300C for 5-6 days and stored in refrigerator at 40C. Sub culturing was carried out once in every 2-3 months.

Solid state fermentation (SSF) and Substrate preparation for cellulase production: Inoculum preparation was carried out using Sabouraud dextrose broth (150 ml), prepared in 500ml Erlenmeyer flasks and autoclaved at 15 lbs for 15 min. The medium was inoculated with isolated fungus of 3 mycelial disc (7 mm diameter) punched out from the edges of its 8 days old colonies from Petri plates. The flasks were incubated at 30 ± 2 °C for 72 hrs. The fungus was inoculated into substrates flasks for enzyme production.

Substrate preparation for cellulase enzyme production: Four different types of agro wastes were used as a substrate for cellulase enzyme production such as wheat bran, sugarcane bagasse, cotton waste and discarded paper. Cotton Waste and discarded paper were pretreated with 3% H_2SO_4. This 2.0 gm of substrates were supplemented to the basal medium. The composition of Basel medium includes KH_2PO_4: 2gm, $(NH4)2PO_4$: 1.4 gm, Urea: 0.3 gm, $CaCl_2.2H_2O$: 0.3 gm, $MgSO_4$: 0.3 gm, Peptone: 1.0 gm, $FeSO_4.7H_2O$: 5.0 mg, $MnSO_4.7H_2O$: 1.6 mg, $ZnSO_4.7H_2O$: 1.4 mg, $CoCl_2.6H_2O$: 2 mg, Tween 80:

0.2% (v/v), D/W 1000 ml and pH: 5.5 (Mandel and Resse, 1957). Substrate flask was incubated at 30°C at 6 pH.

Preparation of crude enzyme: Enzymes were extracted from substrate flask by addition of 5 ml of cold 0.05 M acetate buffer (pH 4.8). The material was filtered through muslin cloth and the filtrate was centrifuged at 5000 rpm at 4°C for 15min. The supernatant was used for determine the enzyme activity such as carboxyl methyl cellulase (CMCase) and filter paper activity (FPase).

Enzyme Assay:Filter paper assay (FPase): According to the method of Mendels and Weber (1969) filter paper activity of the culture filtrates were determined. Whatman filter paper strips containing 50 mg (1cm X 6cm) weight was inoculated in 1 ml of 0.05 M sodium acetate buffer (pH 4.8) and kept at 50 °C in a water bath. 0.5 ml aliquots of enzyme source were added to the above mixture and incubated for 60 minutes at 50°C. After incubation, the released reducing sugar was estimated by the 3, 5-dinitrosalicylic acid (DNSA) method. Control without enzyme was simultaneously run with sample. Activity of cellulase was expressed in filter paper units which were defined as the amount of enzyme releasing 1 mole of reducing sugar from filter paper /ml /min.

Endoglucanases enzyme assay (CMCase): Endoglucanase activity in the culture filtrates was quantified by carboxy-methyl cellulase (CMCase) method as describe by Ghosh (1987). In this method the reaction mixture of 0.5 ml enzyme, 0.5 ml of 1% carboxy methyl cellulose in 0.05 sodium acetate buffers with pH 5.0 and 1ml DNSA were incubated at 50 °C in a water bath for 20 minutes. Appropriate control without enzyme was simultaneously run. The release of glucose due to the enzyme activity was assayed by 3, 5 Dinitro salicylic acid measured at 540 nm using spectrophotometer (Miller, 1959).

Coctail of in house producing enzymes from wheat bran: Four individual cultures (A4, A8, A10, and A11) with their 6 combinations were tested for efficient combination of enzyme in wheat bran under SSF at 30°C. Cellulase enzyme extracted from wheat bran using four cultures were mixed in equal amount and find out their FPase and CMCase activity. Crude enzyme was extracted as above 2.1 methods. Effect of Incubation time, temperature and pH on cellulase enzyme production from wheat bran: Among four different cellulosic waste wheat bran was selected for enzyme production. The influence of temperature (30 to 50 °C), pH (4.0, 5.0, 6.0, 7.0 and 8.0) and incubation period (2-6 days) on enzyme production was studied using A-11 by keeping all other parameters constant. Samples were withdrawn after every 24hr of incubation up to 6 day. The material was filtered through muslin cloth and the filtrate was centrifuged at 5000 rpm at 4°C for 15 min and the supernatant was analysed for FPase and CMCase activity.

RESULTS AND DISCUSSION

Isolation, Primary screening and identification of cellulolytic Fungi: Total 34 different isolates were selected by the primary screening technique from which 4 isolates were showing (Table 1) higher cellulase activity. Potential isolates were obtained from wood furnishing region, paper industry waste and compost wheat bran waste shown- in table 1. In the Primary screening, the isolates were subjected to screening procedure on Carboxy Methyl Cellulose (CMC) plates as described earlier by Narra et al. (2014). After 72 hrs of incubation cellulase activity was measured as a clear zone of hydrolysis. The fungal strains were selected based on cellulose utilization; the zone of hydrolysis was observed after flooding with iodine solution. A4, A8, A10 and A11 were selected for secondary screening of cellulase activity from four different agro wastes (Figure 1).

Table 1. Colony morphology and microscopic observation of selected culture

Isolated Culture	Isolation from	Colony Morphology	Cellulase Zone (mm)
A1	Soil	White to green, dark green spore	7
A2	Cotton waste	White to green, dark green spore	14
A3	Cotton waste	Green to black	12
A4	Vegetable waste	Green to Black, Black mycelium	21
A5	Wood	White to Green, Green spore	18
A6	Cotton waste	White to green, dark green spore	13
A7	Soil	Green to Black	21
A8	Plant	White mycelium, Greyish green	12
A9	Soil	White to green, dark green spore	12
A10	Paper waste	White to Green	18
A11	Wheat bran	White to light Green, light and dark green mycelium	25
A12	Plant	White to light Green	22
A13	Plant	White to green, dark green spore	13
A14	Plant	White to light green, light green mycelium	8

Figure 1: Morphological and Microscopic Observation of Fungi

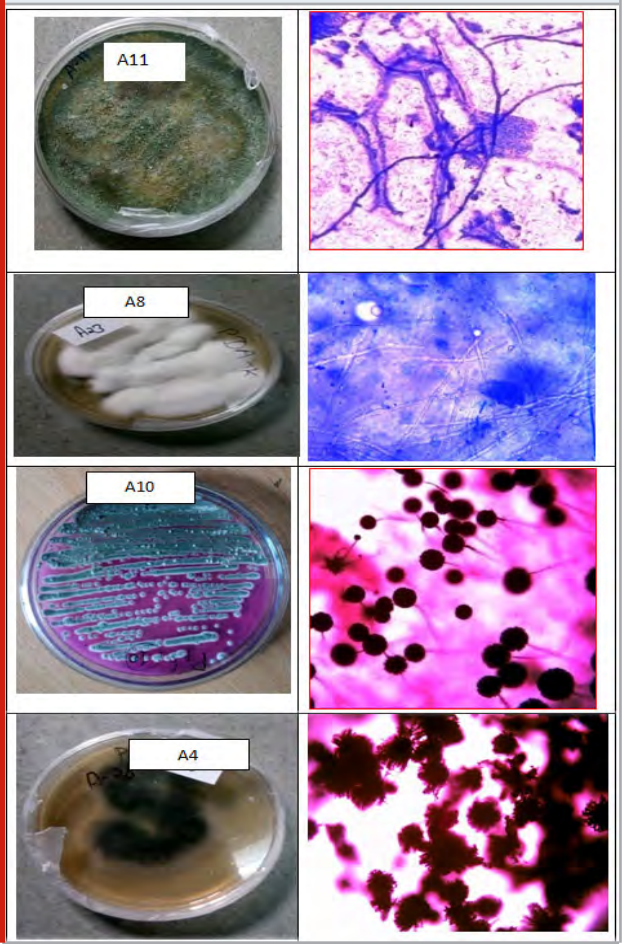


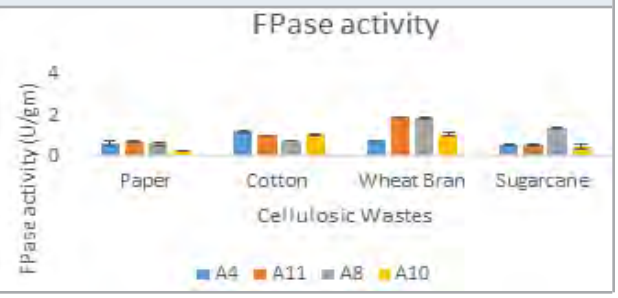
Figure 2A: Inoculum preparation for SSF

Figure 2B: Solid State Fermentation for cellulase production

Solid state fermentation (SSF) for cellulase enzyme production: Inoculum preparation was carried out using Sabouraud dextrose broth. The medium was inoculated with selected fungi and fungi were grown in 72hrs at 30 ± 2 °C (Figure 2A). The fungus was inoculated into substrates flasks for enzyme production (Figure 2B).

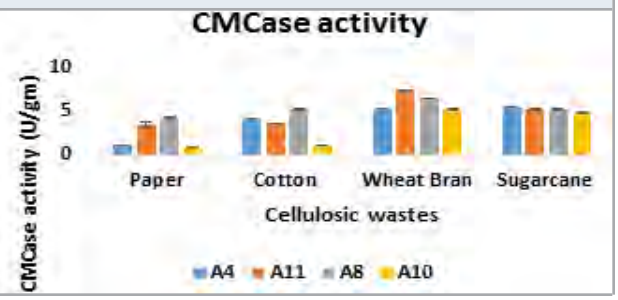
Various agro residues viz. Paper waste, cotton ginning waste, wheat bran and sugarcane bagasse were used for cellulase production by A8, A10, A11 and A4 fungal culture with Mandel's medium as the moistening media under solid state fermentation (SSF). According to Srivastava et al. (2018), Aspergillus and Trichoderma are the main fungal sources of commercial cellulase enzyme production from wheat bran. In the present study optimum FPase activity 1.92U/gm and 1.8U/gm were obtained from wheat bran on 5th day of incubation time at 300C by A11 culture and A8 respectively (Figure 3A).

Figure 3A: FPase activity from cellulosic waste



In case of CMCase activity 7.51 U/gm by A11 and 6.54U/gm A8 were obtained high in wheat bran on 5th day of incubation time at 300C (Figure 3B).

Figure 3B: CMCase activity from cellulosic waste



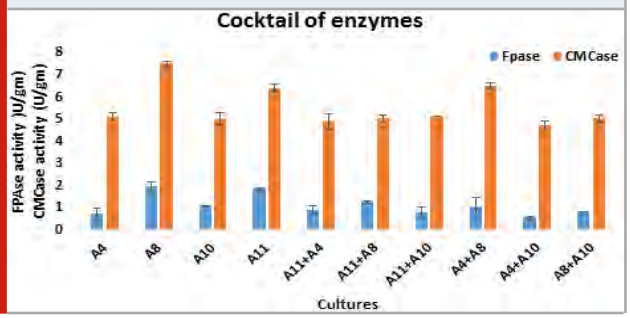
Vyas (2005) have also found that T. viride produced 2 U/ml CMCase and 0.25 U/ml FPase activity on 7th day of incubation. Raghuwanshi et al. (2014) found that CMCase activity: 13.2 IU/g and FPase activity: 2.2 IU/g by Trichoderma from wheat bran on 7th day of incubation. While yield of FPase and CMCase were low with rest of the substrates (Figure 1 and 2). In present study, all selected fungal strains give maximum cellulase activity in wheat bran, which was selected for further cellulase enzyme production.

Earlier it was reported that, Wheat bran is the universal substrate among various substrates because it acts as

a complete nutritious feed for microorganisms having all the ingredients and remains loose even under moist conditions providing a large surface area. It is the need of the time to search for cheaper substrates for cellulase enzyme production so that can be reduced in the cost of biofuels production while as wheat bran is cheap and easily available substrates in India. Furthermore, the biochemical composition of wheat bran indicates that it contains various fermentable sugar such as glucose, xylose, arabinose, galactose, etc. which are helpful for the initiation of growth of microorganisms (Archana and Sathyanarayana, 1997).

Coctail of in house producing enzymes from wheat bran: Four individual cultures with their 6 combinations were tested for efficient combination of enzyme production using wheat bran. The results of FPase and CMCase activity were shown in Figure 3A and 3B respectively. From the result of FPase activity, all the isolates analysed for their monoculture efficiency in enzyme production using wheat bran (Figure 1), it was only A 8 and A11 that had a higher FPase activity (1.98 U/gm and 1.86U/gm respectively) than other mono culture. Co-culture enzyme production was not much better comparing to individual and this shows antagonism effects between cultures (Lequart et al., 1999). Combination of A11 and A8 showed better FPase activity (1.27 U/gm) and combination of A4 and A8 was 6.5U/gm showed higher CMCase activity compare to another enzyme bland. According the Vyas A. (2005) the combination of Aspergillus and Trichoderma showed higher cellulase as well as FPase activity in wheat bran.

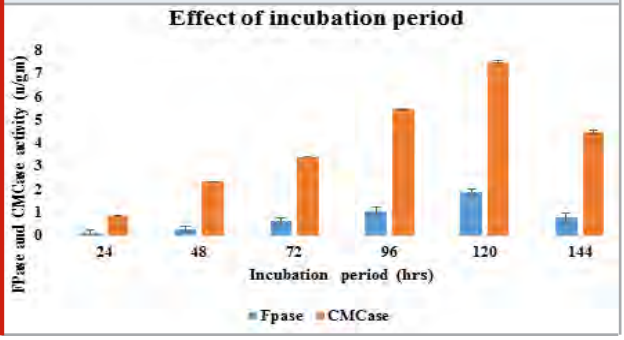
Figure 4: Coctail of in house producing enzymes



Most of the fungi obtained significantly better enzyme production in monocultures which is observed in Figure 4. Maximum CMCase production was found using A8 culture (7.51 U/gm), while A11 gave 6.53 U/gm activity. The cocktail of enzyme also shows better CMCase activity compare to FPase.

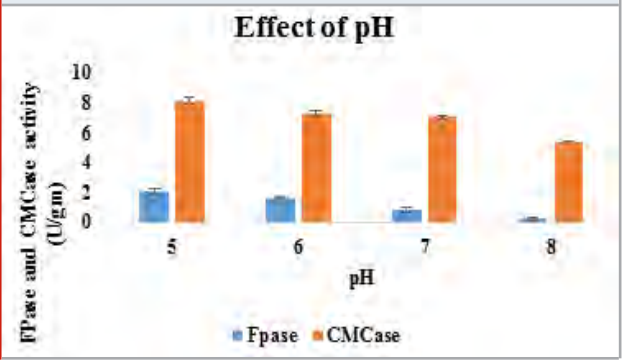
Effect of incubation time on cellulase production: The incubation period directly effects enzyme production. Using SSF A11 (Trichoderma sp.) isolate gave highest enzyme activity of CMCase and FPase 7.51 U/gm and 1.92 U/gm (Figure 5) at 120 hrs of incubation at 30^{0}C temperature which was suitable the commercial point of view. Earlier Gomes I et al. (2006) reported that the cellulase activities were reached to maximal levels within 5-7 days of incubation. After 120 hr cellulase activity was decline it might be due to the depletion of nutrients which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzyme (Nochure et al., 1993).

Figure 5: Effect of incubation time on cellulase production



Effect of Initial pH on cellulase production: pH is an important physical parameter which is effect on the growth rate of fungal strain and significantly affect the enzyme production. Generally, the agro-industrial wastes when used act as a unique buffering action and have an advantage for enzyme production because it has many soluble sugars for growth of microbes. The influence of pH on cellulase production was studied by adjusting the initial medium (Mandal's medium) pH from 4 to 8. The optimum pH for cellulase production was found to be pH 5.0 with an enzyme activity of 2.1U/g of FPase and 8.12 U/gm CMCase using wheat bran by A11 isolates. According to literature's evidence, many researchers reported that acid pH values between 4-6 have favoured cellulase enzyme production (Kuhad et al., 1998). At pH 6.0, 7.0 and 8.0 enzyme activity were found to be 1.67U/g/m, 0.83U/g/m and 0.24U/gm of FPase and 7.34 U/gm, 7.24U/g and 5.4U/gm of CMCase produced respectively (Figure 6).

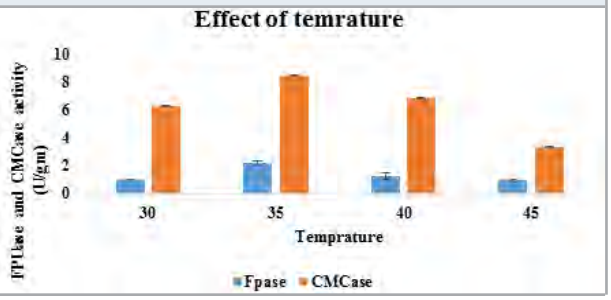
Figure 6: Effect of Initial pH on cellulase production



The enzyme activity was decreased with increasing pH after 5 pH. Highly acidic pH may limit the growth and production by reducing associability of the hemicellulosic substrates, besides that the nature of substrate also has a strong influence on pH kinetics, due to the buffering effect of lignocellulosic substrates.

Effect of Temperature on cellulase production: Temperature has a significant role in the development of biological process as it influences the protein denaturation, enzyme inhibition and cell growth. In the present study the optimum temperature for maximum enzyme production by fungal isolate A11 using Wheat bran as substrate showed was 8.49 U/g and 2.23 U/g activities of CMCase and FPase, respectively recorded at 35°C on 5th day of incubation period (Figure 7).

Figure 7: Effect of temperature on cellulase production



CONCLUSION

In the present study a potential cellulolytic fungal strain *Trichoderma sp*. (A11) was isolated from decomposed wheat bran. Production of cellulases was carried out under SSF at 35°C. The major factors influencing the production of cellulases were found to be temperature, pH, and incubation period. The results revealed that there was an overall increase in FP, β-glucosidase, endoglucanse and exoglucanase activities after the optimization of physical conditions. The newly isolated fungus *Trichoderma sp*. (A11) has the ability to produce cellulase under solid state fermentation in Wheat bran having optimum activity of CMCase 8.49 U/gm and FPase 2.23 U/gm at 120 hrs of incubation, pH 5.0 and at 35°C. This extracted endoglucanase and exoglucanases from *Trichoderma sp*. (A11) has a potential to be utilized for biofuel applications owing to its ability to enhance saccharification of cellulosic waste. Hence the cellulase enzyme obtained in this study hold promise in bioethanol production in bio- processing industrial applications.

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Conflicts of Interest: No potential conflict of interest was reported by the authors.

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Isolation and Molecular Characterization of Arsenic Resistant Bacteria from Brahmaputra River Basin of Assam, India

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ABSTRACT

From the earliest detection of arsenic in the Brahmaputra basin back in 2004, several districts of Assam especially in the flood plains have joined the list of arsenic contamination. Lakhimpur district in Assam India, has been recorded with arsenic concentration several folds higher than the WHO and BIS recommendations. Scientific reports emphasize how native microorganisms can modulate the biogeochemical cycle of arsenic and their possible application in bioremediation. With the aim, this study was designed to isolate and carry out taxonomic characterization of arsenic resistant bacteria from potable water sources of the district. Based on the minimum inhibitory concentration test, two isolates LB6 and NB14 showing the highest resistance were characterized both by biochemical and molecular methods. Morphogenetic characterization identified the strains like *Escherichia coli* -LB6 and Acinetobacter baumannii -NB14. Taxonomic identification was further validated by fatty acid methyl ester analysis. This data of the entire study can conclude that two potential strains E.coli-LB6 and Acinetobacter baumannii-NB14 can resistant arsenic As V (100 to 200mM) and As III (10 to 50mM) concentration in the medium. The results further suggest that strains, LB6, and NB14 can survive under the arsenic stress and has been identified as a potential candidate for application in bioremediation field.

KEY WORDS: ARSENIC, BACTERIA, ASSAM, BRAHMAPUTRA.

INTRODUCTION

Arsenic is a potent human carcinogen. Wide distribution in potable water sources and the negative health impacts of arsenic has raised concerns among scientific societies. Arsenic exists in four valency states in environment viz. arsine (-III), elemental arsenic (0), arsenite (III), and arsenate (V) (Smith et al., 2002). Anthropogenic and natural activities impact its distribution in the aquifer systems. Among the four valency states, arsenite and arsenate are most dominant in a natural environment. Incessant consumption of arsenic-contaminated water can cause skin, lungs, bladder, and kidney cancers (Wang et al., 2018). The maximum contaminant level (MCL) and permissible limit set by the US Environmental Protection Agency (US EPA) and World Health Organization (WHO) is 0.010 mg/L (Hughes., 2002; Shi et al., 2004). This is equivalent to 0.010 parts per million (ppm), 10 micrograms/liter (µg/L), or 10 parts per billion (ppb). Accumulation of arsenic in potable water sources is a serious problem in many parts of the world including India. States of northeastern India were detected with arsenic in several fold higher than the standard permissible level.

Districts like Jorhat, Dhemaji, and Lakhimpur were recorded with arsenic in the range of 300 – 600 µg/L

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(Singh et al., 2004 Das et al., 2015, 2017 Das and Barooah 2018.).

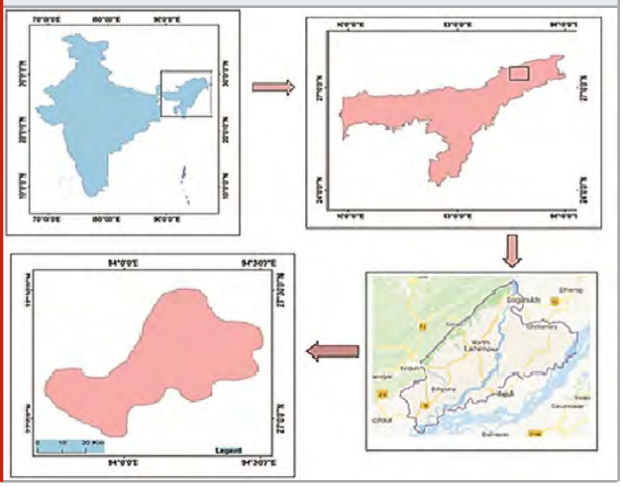
An estimated over 7 million people are using arsenic-contaminated water as a potable source. This alarming fact underlines the importance of this study. Microorganisms are ubiquitous in the environment. They are adapted to environmental extremes. In the evolutionary process, microorganisms had developed an array of metabolic processes to adapt to stressful environments. Microorganisms can actively control the biogeochemical cycles of nutrients and minerals. They are detected in several toxic environments like aluminum (Piña & Cervantes, 1996), cadmium (Ron et al., 1992), uranium (Nevin et al., 2003), cobalt (Sai Ram et al., 2000), mercury (Poulain et al., 2007), lead (Jarosławiecka & Piotrowska-Seget, 2014) and arsenic (Das et al., 2017a). Bacteria can use arsenic in the electron transport chain for energy generation or can oxidize and reduce in a process of homeostasis (Das & Barooah, 2018b, Jihang et al., 2019).

The flexibility of using different elemental compounds in metabolic pathways makes microorganisms as a potential candidate for bioremediation. The innocuous applicability of bioremediation is well documented (Kumar et al., 2018). The involvement of microorganisms in arsenic geocycle is well studied and reported by many researchers (Das & Barooah, 2018; Gnanaprakasam et al., 2017). There is a diverse group of microorganisms that are associated with arsenic biogeochemical cycles (Das et al., 2017a). Microorganisms can oxidize, reduce, and methylate arsenic compounds (Ehrlich.1976; Ahmann et al., 1994; Shariatpanahi et al., 1983). The active association of microorganisms controls the biogeochemical pathway of arsenic. Thus, characterization and identification of microorganisms in the arsenic environment will help us in understanding the inherent community and their association with geocycle of arsenic in the groundwater system. In the present study, two arsenic resistant bacteria were chemotaxonomically characterized and their efficiency of resistance was evaluated. This study will provide a snapshot of the inherent bacterial species of contaminated aquifers of the Brahmaputra river basin and highlight their involvement in arsenic mobilization and speciation.

MATERIAL AND METHODS

Study sites: For the present study, fifty-four water samples were collected from 9 blocks of Lakhimpur district. Samples were collected from tubes, ring wells, and rivers, which are mostly used as potable water sources in the district. Samplings were done in the post-monsoon season (July 2018). Samples were collected in sterilized Nalgene water bottles in replicates. For arsenic analysis, water samples were collected in water bottles pretreated with nitric acid. Collected samples were stored at 40C prior analysis. The locations of the sampling points were obtained with a handheld GPS device (Model: Garmin GPS 72) (Fig. 1).

Figure 1: A cross sectional view of the study area of the Brahmaputra river basin, Lakhimpur district, Assam, India.



Bacterial isolation: For bacterial isolation, 1 ml of water sample was serially diluted and cultured over nutrient agar (NA) (Hi-Media, India) plates containing arsenic at a concentration of As (V) – 1 mg/L and As (III) - 0.5 mg/L. Plates were incubated at 37 0C for 48 h and observed for bacterial growth. Morphologically distinct colonies were selected for further analysis.

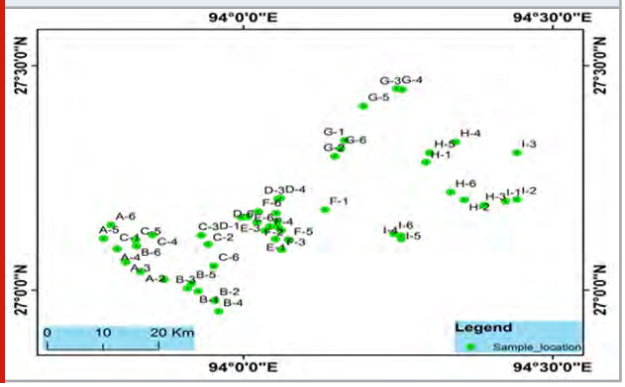
Minimum Inhibitory Concentration: The minimum inhibitory concentration (MIC) of arsenate As (V) and arsenite As (III) was used to determine the arsenic resistance efficiency of the isolates. Bacterial isolates were cultured in freshly prepared NA broth at 30 0C for 48 h and then 100 µl of the freshly cultured bacterial suspension was added to nutrient broth supplemented with different concentration of As(III) (0 – 50 mM) and As(V) (0– 200 mM). Tubes are incubated for 72 h at 30 0C and 150 rpm. Microbial growth was recorded with a UV-Visible spectrophotometer (Himduzu, Japan) at 600 nm.

Morphological and Biochemical characterization: Morphological and biochemical characterization of potential isolates was be done as per Bergey's Manual of Determinative Bacteriology (9th Edn.), Bergey's Manual of Systematic Bacteriology (2nd Edn. 2005). Genomic DNA extraction: Bacterial DNA was extracted from LB6 and NB14 (selected bacteria based on MIC) using QIAGEN (CA, USA) DNA extraction kit. The concentration of DNA was determined using NanoDrop. Fragments of 16S rRNA were amplified using universal primers 27f (16SF-AGAGTTTGATCCTGGCTCAG) and 1492R (16SR-TACGGTTACCTTGTTACGACTT). The PCR product was purified using QIAGEN PCR purification kit and sequenced using ABI 3730 capillary sequencer (16 capillary) with a big-dye terminator reaction.

Sequence analysis and phylogeny: Sequence files were analyzed and quality filtered prior assembly. Sequences were assembled using Codon-Code Aligner (ver 4.0). The assembled sequence was identified using the

nucleotide Blast program against NCBI-nr/nt database and taxonomic profiling was done based on the identity percentage (> 97%). Sequence Alignment has been performed using ClustalW. The evolutionary relationship was determined by a neighbor-joining phylogenetic tree. The base substitution was calculated based on Juke-cantor, one parameter model with 1000 bootstrap values in MEGA 6.0 (Kumar 2013). Identified sequences were compared with the reference sequence from the NCBI nucleotide database.

Figure 2: Sampling sites location of the Brahmaputra river basin, Lakhimpur district, Assam, India.



Fatty acids profiling: The extraction and analysis of the fatty acid methyl ester (FAME) profiles of arsenic resistance bacteria were performed according to the method described by Buyer J. S., (2002). Statistical analysis: All the experiments were done in triplicates and results were presented in mean value with a standard deviation

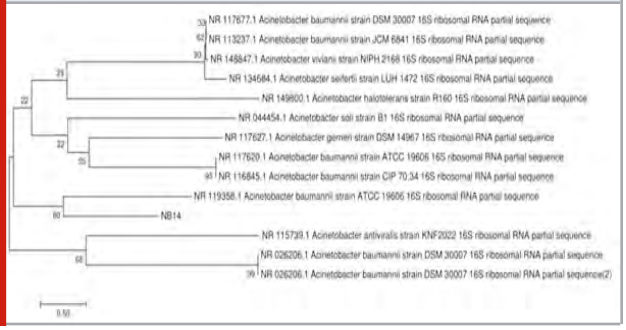
RESULTS AND DISCUSSION

A total of 20 arsenic resistant bacteria were isolated and selected based on morphological distinctness. Arsenic resistant activity and bacterial screening were done by MIC test of different concentration of As (III) and As (V) [Table 1]. It was observed that arsenic affected the growth physiology of all the isolates. Results showed the highest tolerance and growth in isolates LB6 and NB14 in arsenic amended medium compared to control [Table 1]. Other isolates less growth as compared to control and other isolates. Based on MIC value isolate LB6 and NB14 were selected for further study.

Selected isolates LB6 and NB14 showed distinct morphological characteristics [Table 2]. Biochemical characterization showed that isolate LB6 was gram-negative rod-shaped bacteria with positive results catalase, Indole acetate, and H2S production. Isolate NB14 was gram-negative, non-motile, rod-shaped bacteria with positive tests for catalase, H2S production, methyl red, and Voges-Proskauer test. All the biochemical tests and morphological features are presented in [Tables 2 and 3] respectively. Isolated bacteria were taxonomically identified by 16S rRNA genes. Almost complete 16S rRNA gene [LB6 (1486 bp] and NB14 [1462 bp)] was sequenced and the sequence has been submitted to the GenBank database with accession number Escherichia coli-LB-6 [MK332441] and *Acinetobacter baumannii* - NB14 [MK332443]. The comparative analysis of the sequences of isolates with the already available database using BLAST (Basic Local Alignment Search Tool) showed that the strains were close to the other members of the genus. Analysis of the phylogenetic tree sequence indicates that strain LB6 closely related to Escherichia coli U5/41 and NB14 to *Actenobacter viviani* NIPH [Fig 3a and 3b].

Figure 3a: Phylogenetic relationship of LB6 strain with other bacteria

Figure 3.b: Phylogenetic relationship of NB14 with other bacteria



During investigation we have different kind of fatty acids concentration was found in strains *Escherichia coli* -LB-6 and *Acinetobacter baumannii* - NB14 [Result illustrated in table 4]. Major fatty acids detected in E.coli-LB-6 strain were 12:0 (Dodecanoic acid) 4.3%, 14:0 (12-Methyltridecanoic acid) 7.5%, 16:0 (Hexadecanoic acid) 25. 3%, 17:0 (10-Methylene-Hexadecanoic acid) 9.37%. Several reviews have been published in the fatty acid biosynthesis in E coli (Janßen and Steinbüche 2014). Where in *Acinetobacter baumannii* - NB14 strain major fatty acids were 10:0 (3-Hydroydecanoic acid) 8.52%, 13:0 (2-Hydroydodecanoic acid) 8.43%, 16:0 (Hexadecanoic acid) 18.31%. Previously number of fatty acids arachidonic acid (AA) and decosahexaenoic acid (DHA) are highly abundant in *Acinetobacter baumannii* bacterium (Jihang et al., 2019).

A wide range of different fatty acids have been reported from microbial sources. Bacterial fatty acid profile represents the physiological responsiveness to

the surrounding ecological niche it inhabit and the biochemical keys of taxonomical components (Diamonde et al., 2015). Most of microbial source has been viewed as ideal to explore and isolate commercially essential molecules including poly-unsaturated fatty acids (PUFA) (Tonato et al., 2018). This study alone may have little attributes toward taxonomic identity of the strain, but provides important information about molecular signature of the species and contribute to the understanding of the strain's physiological and environmental response to variable ecological niche. Several reports suggest that the fatty acid pool of microorganisms undergo changes in response to the surrounding environment and from strain to strain within a species (Ratledge, 2004).

Table 1. Minimum inhibitory concentration of different isolates

Sr. No.	Isolate code	Control	As(V) concentration (mM)		As (III) concentration (mM)	
		0	100	200	10	50
1	LB1	0.3±0.04	0.12±0.02	0.09±0.02	0.15±0.02	0.05±0.01
2	LB3	0.4±0.02	0.23±0.01	0.15±0.04	0.21±0.05	0.08±0.01
3	LB6	0.81±0.04	0.99±0.02	0.66±0.02	0.87±0.02	0.45±0.01
4	NB-2	0.4±0.03	0.12±0.05	0.14±0.01	0.21±0.04	0.167±0.02
5	NB10	0.54±0.07	0.13±0.01	0.167±0.04	0.31±0.02	0.1±0.05
6	NB-14	0.68±0.03	0.85±0.05	0.84±0.01	0.9±0.04	0.71±0.02
7	NB16	0.64±0.01	0.56±0.03	0.51±0.04	0.34±0.08	0.21±0.8
8	GB3	0.34±0.01	0.21±0.03	0.2±0.04	0.08±0.04	0.06±0.01
9	GB4	0.3±0.05	0.21±0.01	0.11±0.02	0.32±0.07	0.23±0.03
10	BS1	0.53±0.04	0.51±0.02	0.43±0.07	0.21±0.05	0.18±0.05
11	BS2	0.44±0.01	0.4±0.03	0.054±0.08	0.25±0.06	0.20±0.07
12	BS3	0.57±0.03	0.48±0.04	0.43±0.03	0.15±0.08	0.12±0.04
13	DS2	0.43±0.04	0.5±0.04	0.41±0.02	0.21±0.05	0.19±0.04
14	DS4	0.32±0.05	0.26±0.05	0.14±0.07	0.31±0.08	0.27±0.02
15	DS8	0.56±0.03	0.41±0.04	0.36±0.02	0.21±0.03	0.19±0.04
16	HS1	0.5±0.02	0.49±0.03	0.38±0.2	0.34±0.04	0.25±0.03
17	HS6	0.6±0.01	0.34±0.02	0.53±0.08	0.21±0.03	0.11±0.04
18	NS8	0.44±0.01	0.25±0.02	0.18±0.07	0.17±0.08	0.15±0.02
19	NS11	0.52±0.02	0.39±0.03	0.26±0.04	0.35±0.07	0.12±0.08
20	NS19	0.45±0.03	0.22±0.01	0.15±0.01	0.27±0.31	0.14±0.03

Value indicates the mean ± SD of three independent replicates.

Table 2. Microscopic observation of the arsenic resistance, bacterial strains LB6 and NB14

Morphological	LB6	NB14
Colony shaped	Rods	Rods
Color	White	Creamy
Spore	Non-spore forming	Non-spore forming
Gram stains	Negative	Negative
Motility	Motile	Non motile
Surface	Smooth	Smooth
Elevation	Circular	Irregular

Table 3. Biochemical test results for the arsenic resistant bacterial strains LB6 and NB14

Biochemical Test	LB6	NB14
Catalase	Positive	Positive
Oxidation	Negative	Negative
Hydrogen Sulfide Production	Positive	Positive
Indole acetate	Positive	Negative
Methyl red	Positive	Positive
Voges-Proskauer	Negative	Positive
Citrate utilization	Negative	Negative
Glucose	Positive	Negative
Fructose	Negative	Positive
Maltose	Negative	Negative

Microbes play a significant role in the arsenic metabolism pathway. They can transform the different forms of inorganic or organic of arsenic forms As (III) and As (V) undergo oxidation and reduction in our ecosystem (Mukhopadhyay et al., 2002). Several researchers

have reported on isolating arsenic-resistant bacteria from arsenic-rich environments. Besides that different bacterial species such as Caulobacter, Rhizobium and Sphingomonas (Macur et al., 2001) Yersinia intermedia and Yersinia enterocolitica (Bansal et al., 2000), Listeria, Moraxella and Planococcus (Salam et al., 2009), Acinetobacter, Arthrobacter, Agrobacterium, Comamonas, Pseudomonas, Rhodococcus, and Stenotrophomonas (Cai et al., 2009), Bacillus anthracis and Citrobacter freundii (Shakoori et al., 2010), Brevibacillus brevis (Banerjee et al., 2013), Enterobacter asburiae and Enterobacter cloacae (Selvi et al., 2014) Pseudomonas (Satyapal et al .2018) have been reported from different environments site the world.

Table 4. Fatty acid profile of strains LB6 and NB14

FATTY ACIDS	IUPAC/Systemic name	Concentration (%)	
		L B-6	NB-14
10:0	2-Hydroydecanoic acid	-	1.31
10:1	3-Hydroydecanoic acid	-	8.52
11:0	3-Hydroxy-9-Methyldecanoic acid	-	0.36
12:0	10-Methylundecanoic acid	.28	-
12:1	Dodecanoic acid	4.34	5.24
12:0	2-Hydroxydodecanoic acid	-	8.43
12:1	(4Z)-4-Dodecenoic acid	0.43	-
12:2	(8Z)-8-Dodecenoic acid	-	1.65
12.3	3-Hydroxydodecanoic acid		5.58
14:0	12-Methyltridecanoic acid	7.55	.89
14:	11-Methyltridecanoic acid	-	0.14
15:0	(5Z)-13-Methyl-5-Tetradecenoic acid	0.62	0.87
15:1	12-Methyltetradecanoic acid	3.18	1.86
15:2	(10Z)-10-Pentadecenoic acid	3.76	0.56
15:3	(6Z)-6-Pentadecenoic acid	-	0.47
16:0	1-Hexadecanol	0.39	0.29
16:1	14-Methylpentadecanoic acid	0.49	-
16:0	Hexadecanoic acid	25.36	18.31
16:0	10-Methylhexadecanoic acid	0.57	-
17:1 ISO W5C	(12Z)-12-Heptadecenoic acid	3.98	0.81
17:0 ANTEISO	14-Methylhexadecanoic acid	-	1.42
17:1 ANTEISO	(7Z)-13-Methyl-7-Hexadecenoic acid	0.67	0.40
17:0	Heptadecanoic acid	2.71	0.42
17:0 CYCLO	cis-9,10-Methylene-Hexadecanoic acid	9.37	1.57
18:0	16-Methylheptadecanoic acid	4.81	1.70
18:1W7C11-METHYL	(11Z)-10-Methyl-11-Octadecenoic acid	1.29	-
18:3W6C	12Z)-12-Octadecenoic acid	-	1.15
18:1W5C	(13Z)-13-Octadecenoic acid	-	0.29
19:0CYCLOW8C	cis-11,12-Methylene-Octadecanoic acid	4.35	-
19:0	Nonadecanoic	1.20.	-
20:0 ISO	Icosanoic acid	0.38	0.80
20:2W9C	(11Z)-11-Icosenoic acid	0.78	
20:2W6C	(11Z,14Z)-11,14-Icosadienoic acid	-	0.30

In the present study, we have a 20th isolates isolated from water samples Brahmaputra river basin. The 20 isolates were screen in the based on ability to grow on high levels of As (III) and As (V) containing medium. All of the isolates examined in this study were found to be resistant to both As (III) 50mM and As (V) 200mM of concentration in the medium, respectively. Out of them, we have selected two potential isolates LB6 and NB14 based on MIC data. They were found both are high resistant up to As V (100 to 200mM) and As III (10 to 50mM) in the medium. After morphological, biochemical, and 16 rDNA sequence molecular characterization we have confirmed that LB6 strain was *Escherichia coli* and NB14 strain was *Acinetobacter baumannii*. *Escherichia*

coli and *Acinetobacter* broadly represent arsenic resistant bacteria strain isolated from an arsenic-rich environment (Anderson and Cook 2004; Jackson et al., 2005).

A bacterium as we know *E. coli* is a model microorganism. A huge amount of research has been fields like biotechnology and molecular levels. Similar result reported the Escherichia coli, which are resistant up to 909.79 mg/L As (IV) and 3120.1 mg/L As (III) (Bista and Shakya 2017), Acinetobacter baumannii, which are resistant up to (40 mM to 300 mM As V) and As III (4mM to 25mM As III) reported by (Alaniz et al., 2017). *Staphylococcus sp*. TA6 was isolated from arsenic contamination sites of groundwater of Jorhat, Assam, and biotransformation of arsenate to arsenite. They suggested the potential isolates will play important role in arsenic geo cycle in Brahmaputra valley (Das & Barooah, 2018b).

A similar study reported the two arsenic resistant bacteria *Bacillus sp*. and *Aneurinibacillus aneurinilyticus*, which can arsenic resistant up to As (V) 4500 ppm and As (III) 550 ppm isolated from arsenic affected groundwater of Purbasthali block of Burdwan, West Bengal, India, (Dey et al., 2016). Another study reported the *Bacillus* and Geobacillus arsenic oxidizing bacterial strain isolated from arsenic-contaminated soil of West Bengal, India. They were found both strains were As III (16-47mM) and As V (167-400mM) concentration is resistant in medium (Majumder et al., 2013). In northeastern India, the presence of arsenic has been identified in 21 districts out of 24 districts of Assam and six in Arunachal Pradesh, one in Manipur, three districts in Tripura, and two in Nagaland (Singh 2004; Mukherjee et al., 2006).

CONCLUSION

The extent, distribution, origin, and mobilization process of arsenic and iron in the aquifers of river Brahmaputra basin, mostly located in the Indian state of Assam, has been largely undocumented, and unexplored. Moreover, the river Brahmaputra basin has a boundary of tea gardens and paddy fields where a huge amount of fertilizers and pesticides are being used. The arsenic forms weak bonds with certain organic material, helps the arsenic to precipitate which may lead to arsenic contamination in groundwater and further decomposed in the same land, concentrating it. This entire study can conclude two potential strains were *E.coli*-LB6 and *Acinetobacter baumannii*-NB14 can resistant arsenic As V (100 to 200mM) and As III (10 to 50mM) concentration in the medium. The results suggest that strains, LB6, and NB14 can survive under the arsenic stress and has been identified as a potential candidate for application in bioremediations field.

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The Exposure to Traumatic Experiences Among the Palestinian Students in the West Bank

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ABSTRACT

The current study aims to investigate the long-term effects of war and occupation among Palestinian children in West Bank.The situation in the West Bank is uncommon in the frequency with which children are exposed to war-related traumatic events on a daily basis and because of the long-term nature of the conflict. The study sample was basic school students in West Bank; that consisted of 537 students; 242 (45%) were males and 295 (55%) were females and the mean of age in the sample was (14.8 ± 1.12). There were 341 (64%) of the students from villages and there were 196 (36%) students from cities. The participants completed a Checklist of Traumatic Experiences (CTE).The study found that almost every Palestinian child of the sample had been exposed traumatic experiences (chronic trauma). There is more than 22% of the participants exposes from 11 - 15 traumatic experiences from the total 34 traumatic experiences; such as any of your friends, neighbours, or relatives been injured by the occupying forces, inhaling tear gas, any of your friends, neighbours, or relatives been killed by occupying forces, witnessed anyone being arrested by the occupying forces, and the occupied forces used your house, block, camp, or zone as a cordon. Also, the study found that males are more exposed to traumatic experiences than females; moreover, there were significant differences between residences; students from villages are more exposed to traumatic experiences than cities.The study provides valuable evidence that demographic and socioeconomic factors mediate the relationship between different war traumatic events. Interventions should take into account the children's background including their gender, age, where they live, and their socioeconomic status (e.g., family income, parents' educational level, family size) to alleviate the psychological symptoms and to enhance their resilience.

KEY WORDS: TRAUMATIC EXPERIENCES, YOUTH, PALESTINE, COLLECTIVE TRAUMA, CTE.

INTRODUCTION

For more than half a century, Palestinians have suffered from various levels of traumatic experiences. Since the beginning of the second Intifada, which began in September 2000, the Palestinian people have been exposed to violence. The Palestinian nation suffers from traumatic events imposed by armed and/or military violence together with restriction of movement through checkpoints, closures and curfews. Traumatic events such as shootings, bombings, destruction of houses, fields, physical violence and deaths occur on a daily basis, El-Khodary Samara 2019a & 2019b).The Palestinian children who are part of the society living under occupation suffer from insomnia, fear of the dark, phobias, depression, bedwetting, social withdrawal, negative social-interaction, aggressive behaviour, forgetfulness and truancy from school. These indicators reveal that it is almost impossible to have a normal childhood in Palestine under the current circumstances and it is affecting their future psychological well-being (Altawil, 2008, El-Khodary Samara 2019a & 2019b).

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Gaboulaud et al. (2010) presented data of 1773 children and adults who received treatment by psychotherapists between November 2000 and January 2006, in the Gaza Strip and in the West Bank. Nearly half of the patients were children between 4 and 14 years. The three main diagnoses were a) anxiety disorder, b) mood disorder, and c) PTSD.In addition, most of the studies regarding psychological health and recovery in Palestine were conducted in the Gaza Strip. The results have revealed that Palestinian children who live in war zones are at high risk of suffering from PTSD, somatic disorders and psychosocial problems (Kanninen, Punamäki, & Qouta, 2003; Qouta & El-Sarraj, 2004; Thabet & Vostanis, 2000). Palestinian students who grew up in the Intifada depicted students in their drawings as being beaten or shot by soldiers (Garbarino, Kostelny, & Dubrow, 1991; Holt, 2001). Furthermore, the number of traumatic experiences was related to higher levels of neuroticism and the lack of attention, concentration and memory (Qouta, Punamäki, & Sarraj, 1995).

A study by Abu Hein, Qouta, and El Sarraj (1993) found a high rate, about (25%), of the Palestinian students that were living in Gaza strip they were exposed to traumatic experiences during the first intifada. Another study revealed that Palestinian children who living in the West Bank they were mainly suffer from behavior and psychosomatic problems (Baker, 1990). Therefore, it seems that Palestinian children are surviving from traumatic events. They need to stand up, adapt, bounce back, recover and endeavor to overcome all difficulties in spite of the circumstances that surround them. Given that the majority of the people are exposed to traumatic events, the question is not only the type of oppression from which they suffer, but how to foster the capacity to overcome such difficult circumstances. Researchers and psychologists have emphasized disappointment and unhappiness as well as anxiety and depression rather than the strengths and potentialities of the people of Palestine, but still the main question remains how to facilitate overcoming traumas or how to grow up with a good mental health in spite of the traumatic events. (El-Khodary Samara 2019a & 2019b).

The Diagnostic and Statistical Manual of Mental Disorders, 4th edition, Text Revision (American Psychiatric, 2000), defines trauma including the events, and the person response to it. Trauma as direct personal experience of an event that involves actual or threatened death or serious injury, or other threat to one's physical integrity; or witnessing an event that involves death, injury, or a threat to the physical integrity of another person; or learning about unexpected or violent death, serious harm, or threat of death or injury experienced by a family member or other close associate (Criterion A1). And criterion A2, as the person's response to the event must involve intense fear, helplessness, or horror (or in children, the response must involve disorganized or agitated behavior) (Criterion A2). (American Psychiatric, 2000).

Several studies have revealed that exposure to previous traumatic war experiences and events is a risk factor for the development of post-traumatic stress disorder (PTSD), grief, and depression. The exposure to traumatic events, specifically physical injuries, loss of loved ones, immediate risk of life, injury of a family member or friend and losing a family member are the strongest risk factors for PTSD (El-Khodary Samara 2019a & 2019b). Individuals or groups exposed to traumatic experiences generally demonstrate some form of stress that is why it is often referred to as traumatic stress. Responses of Traumatic stress has been widely researched by a psychologists; (Awadh, Vance, El-Beblawi, & Pumariega, 1998; Barber, 2009; Benjamin & Crawford-Browne, 2010; Bonanno, 2004; Breslau, Davis, & Andreski, 1995; Daniel, Jane, & Ann, 2005; Elbedour, Onwuegbuzie, Ghannam, Whitcome, & Hein, 2007; Espié et al., 2009, Palosaari, Punamäki, Diab, & Qouta, 2013; Stevens, Eagle, Kaminer, & Higson-Smith, 2013; Thabet, Abu Tawahina, El Sarraj, & Vostanis, 2008; Thabet & Vostanis, 1999; Yule, 2000 Dimitry, 2012; Dubow et al., 2012; Eagle & Kaminer, 2013; Khamis, 2015).

Post traumatic Stress Disorder (PTSD) is included in the DSM-5 on a new chapter called Trauma and Stress or Related Disorders, (American Psychiatric, 2013).The diagnostic criteria for the manual's next edition identify the trigger to PTSD as exposure to actual or threatened death, serious injury or sexual violation. The exposure must result from one or more of the following scenarios, in which the individual experiencing the traumatic event in a direct way, witnessing the traumatic event personally,and an indirect experience of the traumatic event that occurred to a close family member or close friend (with the actual or threatened death being either violent or accidental); or immediate repeated experiences or extreme exposure to cruel forms of the traumatic event (not through media, unless work-related). (American Psychiatric, 2013).

However, traumatic stress does not necessarily lead to PTSD or other mental disorders; in fact, the majority of cases resolve themselves over time and does not create any lasting psychopathology (J. Breslau, 2004). This is especially important given that evidence demonstrates that cross-cultural differences exist in the manner by which emotional and behavioral disorders and problems are expressed (Rahman, Mubbashar, Harrington, & Gater, 2000). Moreover, in situations of war and conflict, violence and trauma are often experienced collectively, with repercussions for a sense of community security, and not merely individually (Giacaman, Shannon, Saab, Arya, & Boyce, 2007).

In this regard, some scholars have also attempted to resolve this disjuncture by harmonizing different perspectives of individual and collective trauma (e.g. Abramowitz, 2005; Kienzler, 2008)). In response to this, new dimensional approaches to trauma are being developed, which integrate the biological, cultural and clinical dimensions of trauma in the explanatory framework of trauma (e.g. Kirmayer et al., 2007). There is a need to assess if Palestinian children live under

traumatic situations. Individuals who are directly or indirectly exposed to war and conflict experience a variety of adverse short and long-term psychological reactions. Common symptoms and reactions in the aftermath of potentially traumatic experiences include anger, sleeping difficulties, nightmares, and avoidance of situations that are reminders of the trauma, impairment of concentration, and guilt due to survival or lack of personal injury during the traumatic event. A number of studies have found a high prevalence of symptoms, including Post Traumatic Stress Disorders (PTSD) among children exposed to war trauma, state-sponsored terrorism or interpersonal violence (Palestinian Center for Human Rights, 2009).

Table 1. Exposure to traumatic experiences: frequency and percentage of traumatic experiences

#	The items of traumatic experiences	yes	%
24	Have you been exposed to the hearing of the explosion sounds or the sound bombs?	429	.7989
3	Have you been exposed to inhaling tear gas?	384	.7151
29	Have you witnessed a martyr's funeral?	344	.6406
32	Have you witnessed anyone being arrested by the occupying forces?	311	.5791
11	Have the occupied forces used your house, block, camp, or zone as a cordon?	287	.5364
30	Have you witnessed the occupying forces beating anyone?	278	.5177
15	Has any of your friends, neighbours, or relatives been killed by occupying forces?	277	.5158
31	Have you witnessed injuring by the occupying forces?	258	.4804
27	Have you witnessed the occupying forces opening fire against people?	250	.4655
17	Has any of your friends, neighbours, or relatives been injured by the occupying forces?	241	.4488
20	Have you attended to martyr's funeral?	214	.3985
19	Has anyone been killed in front of your eyes by occupying forces?	206	.3836
33	Have you witnessed the occupying forces destroying trees or farms?	169	.3147
34	Have you witnessed the occupying forces not allowing an ambulance to reach a hospital?	167	.3110
16	Has any of your close family members been injured by occupying forces?	160	.2980
23	Have the occupied forces destroyed a land or farm of yours or of a dear person by a bulldozer.	142	.2644
14	Has any of your close family members (father, mother, brother, sister) been killed by occupying forces?	126	.2346
22	Has anyone of your close family members been exposed to humiliation by occupying forces?	122	.2272
25	Have you witnessed the occupying forces destroying house(s).	108	.2011
28	Have you witnessed people being shelled and bombed?	105	.1955
8	Have you been exposed to live fire by occupying forces, but you were not injured?	100	.1862
7	Have you been injured to the degree that you lost consciousness?	82	.1527
12	Have the occupied forces threatened you with the possibility of not allowing access to your home?	78	.1453
21	Have you been exposed to humiliation by occupying forces?	71	.1322
26	Have you witnessed shelling by tanks, artillery, or military planes?	63	.1173
18	Has anyone of your close family members been killed in front of your eyes by occupying forces?	57	.1061
9	Have you been exposed to shelling by tanks, artillery, or military planes, but you were not injured?	56	.1043
10	Have you been beaten by occupied forces?	34	.0636
6	Have you been shot with a rubber bullet by occupying forces?	33	.0615
4	Have you been injured by shelling (e.g. wounds, burns, or bone break) by tanks, artillery, or military planes?	32	.0596
2	Has your house been partially destroyed by shelling or bulldozing?	22	.0410
13	Have you been arrested by occupying forces?	18	.0336
1	Has your house been completely destroyed by shelling or bulldozing?	16	.0298
5	Have you been shot with live ammunition by occupying forces?	9	.0168

Research have been mainly done in Gaza, and few data are coming from the West Bank; assessing traumatic experiences is not only to assess PTSD, but also checking how everyone perceive their situation according to their especial context, and specially under a collective traumatic situation. These information will allow to take measures to increase not only individual psychotherapeutic attention, but community psycho-social attentions in war torn zones (Shalhoub-Kevorkian, 2008). The war and the long term occupation of Palestinian territory

expose students to recurrent traumatic experiences which violate their human rights: the right to live, to learn, to be healthy, to live with his/her family and community, to develop his/her personality, to be nurtured and protected, and the right to enjoy childhood. The potential for having a normal childhood in Palestine is unlikely in the current circumstances and the future psychological well-being of Palestinian children is at risk of being compromised by on-going traumatic experiences.

MATERIAL AND METHODS

Participants and Procedure: The sample consisted of 537 Palestinian public-school students of 13 and 14 years old living in the West Bank (OPT Occupied Palestinian Territories). They were 55% girls and 45% boys. About two thirds (64%) were from rural areas and (36%) from urban areas. For the study, 25 schools were randomly selected as representative of schools in the North directorate of the West Bank. At each school 10 students from 8th grade and 10 students from 9th grade, were randomly selected. The High Ministry of Education provided the permission to access the public schools, and then researcher informed the pupils, their parents, and headmaster about the purpose of study, obtaining their consent for participation. Measures:Traumatic war experiences: The Checklist of Traumatic Experiences (CTE) (Altawil, 2008), is a 34-item scale that covers events that are typical in the Palestinian Israel-occupied territories, such as being arrested, threated, injured, exposure to shelling or house demolition. Adolescents reported whether they had been exposed to the events (Yes = 1; No = 0), and the number of times that they have experienced such event. In this study, each item has been analysed individually, taking the frequency that students reported that they have experienced each situation. Frequencies reported higher than 10 times were recoded as 10.

RESULTS AND DISCUSSION

Research findings presented in this study, contain two parts; the first part presents descriptive statistics of Exposure to traumatic experiences: frequency and percentage of the traumatic experiences. The second part presents descriptive statistics of constructs exposure to traumatic experiences, according to the effects of Demographic Factors as gender, place and area.

First part: How Palestinian adolescents are exposed to traumatic experiences, data shows that all of the forms suffered by the sample, being the most often the explosions and sound bombs, tear gas, and funerals; while the lowest is being shouted or house being bulldozed (see table 1, 2 & 4)

According to the dimensions of traumatic experiences, the researcher found that the most dimension exposed among the respondents was the Distant exposure with; (M: 0.42, SD: 0.30) and the least dimension was Material Exposure with; (M: 0.11, SD: 0.18), (see table 4, and Figure 1).

Table 2. Number of traumatic experiences and percentages according to the types (N: 537)

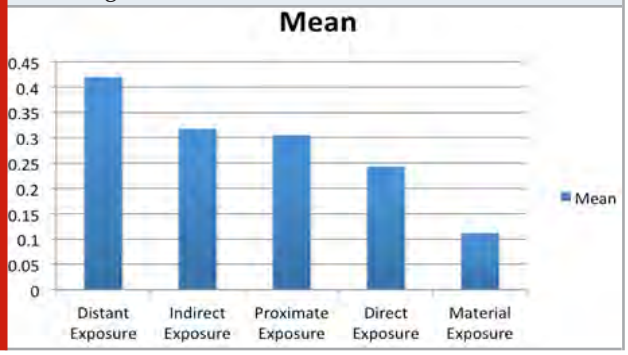
Types	N. of Traumas	Frequency	Percent %	Valid Percent	Cumulative Percent
F	0-5	134	25.0	25.0	25.0
E	6-10	183	34.1	34.1	59.0
D	11-15	120	22.3	22.3	81.4
C	16-20	82	15.3	15.3	96.6
B	21-25	14	2.6	2.6	99.3
A	26-34	4	.7	.7	100.0
Total		537	100	100.0	

Table 3. The means of exposure to traumatic events according to the dimension, in descending order

Dimension		N	Range	Minimum	Maximum	Mean	Std. Deviation
V	Distant Exposure	537	1.00	.00	1.00	.4195	.30115
III	Indirect Exposure	537	1.00	.00	1.00	.3175	.26579
IV	Proximate Exposure	537	1.00	.00	1.00	.3051	.24216
I	Direct Exposure	537	1.00	.00	1.00	.2431	.15587
II	Material Exposure	537	1.00	.00	1.00	.1117	.17964

Second part: this part of the Effects of Demographic Factors will answer the question if there are significant differences in the level of exposure to traumatic experiences according to (gender, place, age and Area). In order to test the differences between the respondents in study variables, according to gender, place, and Age the researcher used independent sample t-test. Table 6 shows the results.

Figure 1: The means of exposure to traumatic events according to the dimension



As illustrated in table 6, there are significant differences between males and females in CTE in benefit to males ($p < 0.05$), also there are significant differences between Cities and Villages in CTE in benefit to Villages ($p < 0.05$). And no significant differences according to the Age.In order to test the differences between the respondents according to Gender, the researcher used one-way ANOVA test, tables 7 and 8 show the results.

There are significant differences between males and females in the dimension; Direct Exposure, Indirect Exposure, Distant Exposure and CTE total in benefit to males ($p < 0.01$). Thus the males are more likely to expose to traumatic experiences than female. In order to test the differences between the respondents according to Place the researcher used one-way ANOVA test, tables 9 and 10 show the results.

Table 4. Frequency and percentages of traumatic experiences (N: 537)

The statements of traumatic experiences	Item no	Type	Frequency	(%)
Has any of your friends, neighbors, or relatives been injured by the occupying forces?	17	IV	242	.4488
Have you witnessed the occupying forces opening fire against people?	27	V	250	.4655
Have you witnessed injuring by the occupying forces?	31	V	258	.4804
Has any of your friends, neighbors, or relatives been killed by occupying forces?	15	IV	277	.5158
Have you witnessed the occupying forces beating anyone?	30	V	278	.5177
Have the occupied forces used your house, block, camp, or zone as a cordon?	11	I	287	.5364
Have you witnessed anyone being arrested by the occupying forces?	32	V	311	.5791
Have you witnessed a martyr's funeral?	29	III	344	.6406
Have you been exposed to inhaling tear gas?	3	I	384	.7151
Have you been exposed to the hearing of the explosion sounds or the sound bombs?	24	I	429	.7989

Table 5. The Results of Independent Sample T-Test for the Differences in Study Variables According to Gender, Place, and Age

Constructs	Mean	S.D.	Mean	S.D.	T-value	P-value
Gender	Males n = 242		Females n = 295			
CTE	0.321	0.17	0.260	0.157	3.99**	0.000
Place	Cities n = 196		Villages n = 341			
CTE	0.263	0.162	0.301	0.166	-2.95**	0.009
Age	Age 13 n = 268		Age 14 n = 269			
CTE	0.31	0.19	0.34	0.21	-1.55	0.120

There are significant differences between respondents from cities and villages in the dimension; Direct Exposure, Indirect Exposure, and CTE total in benefit to villages ($p < 0.01$). Thus the respondents from villages are more likely to expose to traumatic experiences than respondents from cities. The differences between the respondents according to areas (Directorates) the researcher used one-way ANOVA test and tables 11 and 12 show the results.

The result of one-way ANOVA test shows there are significant differences among respondents in exposing to traumatic experiences according to directorate (CTE $F = 13.82$, $p < 0.01$). The most significant finding in this

study was that a high proportion of Palestinian children reported that they had exposed to traumatic experiences; Most of participants had been exposed to all 34 traumatic experiences, there are more than 22% of the participants exposes at least to 15 traumatic experiences from the total of 34 traumatic experiences.

Table 6. The Results of Descriptive statistics For the Study Variables According to Gender

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Direct Exposure	Male	242	.2863	.16905	.01087	.2649	.3077	.00	.93
	Female	295	.2075	.13287	.00774	.1923	.2227	.00	.71
	Total	537	.2430	.15516	.00670	.2299	.2562	.00	.93
Material Exposure	Male	242	.1253	.19536	.01256	.1006	.1501	.00	1.00
	Female	295	.1006	.16513	.00961	.0816	.1195	.00	.67
	Total	537	.1117	.17964	.00775	.0965	.1270	.00	1.00
Indirect Exposure	Male	242	.3502	.26072	.01676	.3172	.3832	.00	1.00
	Female	295	.2907	.26734	.01556	.2600	.3213	.00	1.00
	Total	537	.3175	.26579	.01147	.2950	.3400	.00	1.00
Proximate Exposure	Male	242	.3202	.23607	.01518	.2904	.3501	.00	.83
	Female	295	.2927	.24675	.01437	.2644	.3209	.00	1.00
	Total	537	.3051	.24216	.01045	.2846	.3256	.00	1.00
Distant Exposure	Male	242	.4604	.30016	.01930	.4224	.4985	.00	1.00
	Female	295	.3860	.29828	.01737	.3518	.4201	.00	1.00
	Total	537	.4195	.30115	.01300	.3940	.4451	.00	1.00
CTE Total	Male	242	.3215	.16967	.01091	.3000	.3429	.00	.82
	Female	295	.2596	.15705	.00914	.2416	.2776	.00	.76
	Total	537	.2875	.16559	.00715	.2735	.3015	.00	.82

Table 7. The Results of One Way ANOVA for the Differences in Study Variables According to Gender

		Sum of Squares	df	Mean Square	F	Sig.
Direct Exposure	Between Groups	.825	1	.825	36.564*	.000
	Within Groups	12.078	535	.023		
	Total	12.904	536			
Material Exposure	Between Groups	.082	1	.082	2.537	.112
	Within Groups	17.214	535	.032		
	Total	17.296	536			
Indirect Exposure	Between Groups	.471	1	.471	6.740*	.010
	Within Groups	37.394	535	.070		
	Total	37.865	536			
Proximate Exposure	Between Groups	.101	1	.101	1.728	.189
	Within Groups	31.332	535	.059		
	Total	31.433	536			
Distant Exposure	Between Groups	.738	1	.738	8.244*	.004
	Within Groups	47.871	535	.089		
	Total	48.609	536			
CTE Total	Between Groups	.508	1	.508	19.171*	.000
	Within Groups	14.189	535	.027		
	Total	14.697	536			

The study found that boys suffer more traumatic experiences than girls, which is similar to the findings of many previous studies (Husain et al., 1998; Khamis, 2005; Thabet, Tawahina, El Sarraj, & Vostanis, 2008; Thabet & Vostanis, 1999) (Kuterovac, Dyregrov, & Stuvland, 1994). However, it contrasts with another study that found that boys and girls in Palestine, both of them have the same level of traumatic experiences (Miller, El-Masri, Allodt, & Qouta, 1999, El-Khodary, Samara 2019a & 2019b), which lead us to understand the difference between traumatic events in Gaza and in the West Bank.

Table 8. The Results of Descriptive statistics For the Study Variables According to Place

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Direct Exposure	Village	341	.2570	.15735	.00852	.2403	.2738	.00	.79
	City	196	.2187	.14854	.01061	.1977	.2396	.00	.93
	Total	537	.2430	.15516	.00670	.2299	.2562	.00	.93
Material Exposure	Village	341	.1163	.18210	.00986	.0969	.1357	.00	1.00
	City	196	.1037	.17544	.01253	.0790	.1285	.00	.67
	Total	537	.1117	.17964	.00775	.0965	.1270	.00	1.00
Indirect Exposure	Village	341	.3468	.26530	.01437	.3185	.3750	.00	1.00
	City	196	.2666	.25952	.01854	.2300	.3031	.00	1.00
	Total	537	.3175	.26579	.01147	.2950	.3400	.00	1.00
Proximate Exposure	Village	341	.3123	.24647	.01335	.2861	.3386	.00	1.00
	City	196	.2925	.23456	.01675	.2595	.3256	.00	.83
	Total	537	.3051	.24216	.01045	.2846	.3256	.00	1.00
Distant Exposure	Village	341	.4349	.29809	.01614	.4031	.4666	.00	1.00
	City	196	.3929	.30533	.02181	.3498	.4359	.00	1.00
	Total	537	.4195	.30115	.01300	.3940	.4451	.00	1.00
CTE Total	Village	341	.3015	.16632	.00901	.2838	.3193	.00	.79
	City	196	.2631	.16184	.01156	.2403	.2859	.00	.82
	Total	537	.2875	.16559	.00715	.2735	.3015	.00	.82

Table 9. The Results of One Way ANOVA for the Differences in Study Variables According to Place

		Sum of Squares	df	Mean Square	F	Sig.
Direct Exposure	Between Groups	.183	1	.183	7.702*	.006
	Within Groups	12.721	535	.024		
	Total	12.904	536			
Material Exposure	Between Groups	.020	1	.020	.610	.435
	Within Groups	17.276	535	.032		
	Total	17.296	536			
Indirect Exposure	Between Groups	.800	1	.800	11.553*	.001
	Within Groups	37.065	535	.069		
	Total	37.865	536			
Proximate Exposure	Between Groups	.049	1	.049	.832	.362
	Within Groups	31.384	535	.059		
	Total	31.433	536			
Distant Exposure	Between Groups	.220	1	.220	2.427	.120
	Within Groups	48.390	535	.090		
	Total	48.609	536			
CTE Total	Between Groups	.184	1	.184	6.794*	.009
	Within Groups	14.513	535	.027		
	Total	14.697	536			

Table 10. The Results of Descriptive statistics For the Study Variables According to Directorate

Construct	Directorate	N	Mean	S.D.
CTE	Jenin	82	0.37	0.21
	Qabatya	45	0.45	0.23
	Nablus	132	0.24	0.17
	S. Nablus	43	0.44	0.15
	Salfit	40	0.41	0.18
	Tubas	26	0.25	0.20
	Tulkarm	103	0.27	0.18
	Qalqilya	66	0.38	0.17

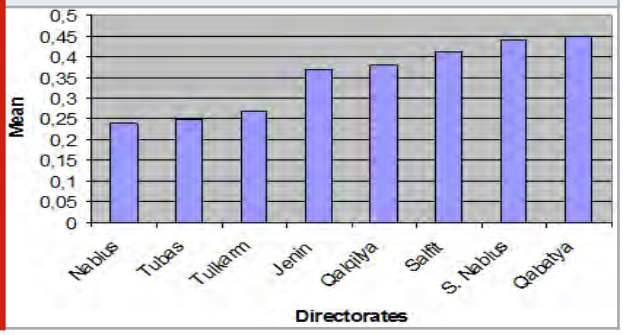
In Gaza we find a general bombing which facilitates the exposure and suffering for everyone, including children, while in the West Bank traumatic events are mostly found in some specific areas; check points, some villages and even houses, as the exposure cannot be experienced by all the population.The results found that the percentage of exposure to traumatic experiences increase for those whom living in the villages than those whom living in the cities, specially in the dimension; Direct Exposure and Indirect Exposure to traumatic events. This is consistent with previous studies (Thabet & Vostanis, 2000; Thabet, Abed, & Vostanis, 2004; Thabet & Vostanis, 2019).

According to Areas or directorate, there are more exposure to traumatic events on some areas like Qabatya, south Nablus and Salfit, These directorates include many villages and communities as well as their closeness to settlements, military checkpoints, and the apartheid wall, all of these factors might be leading to increase clashes with settlers and soldiers, therefore increased exposure to traumatic events.The war and the long term occupation of Palestinian territory expose students to recurrent traumatic experiences which violate their human rights: the right to live, to learn, to be healthy, to live with his/her family and community, to develop his/her personality, to be nurtured and protected, and the right to enjoy childhood. The potential for having a normal childhood in Palestine is unlikely in the current circumstances and the future psychological well-being of Palestinian children is at risk of being compromised by on-going traumatic experiences.

Table 11. The Results of One Way ANOVA for the Differences in Study Variables According to Directorate

Directorate	Source of variance	Sum of Squares	df	Mean Square	F	Sig.
CTE	Between Groups	3.339	7	0.477	13.815**	0.000
	Within Groups	18.265	529	0.035		
	Total	21.604	536			

Figure 2: The Results of Descriptive statistics For the Study Variables According to Directorate



CONCLUSION

Results show that childhood in the West Bank suffers from traumatic situations; in addition to that the results, which come from Gaza, are worst. Therefore, intervention must go on two directions: 1) stop oppression and aggressive acts that provoke traumatic experiences, and 2) increasing the resistance and resilience of the oppressed population. The current study proves that the surrounding environment of the child has an influence on the development of many kind of mental disorders either as a risk or as a protective factor. The application of the ecological framework theory with children exposed to difficult situations as here involve the relationships between risk and protective factors in the various levels of the ecological model which are the individual (e.g., age, gender), family (e.g., family size, SES), and environment (type and place of residence, citizenship, war trauma and political situation) (El-Khodary Samara & Askew 2020).At the end, this paper showed how adolescents are affected by Israel occupation, assessed with a questionnaire of traumatic experiences checklist show that the situation is hard, but still bearable comparing to Gaza. This means that oppressed and occupation forces can oppress even more, and also that recovering is easier.Nevertheless, this paper brings the most important point the need to develop appropriate tools to assess traumatic experiences or aggression to adolescents, taking into consideration if the aggression is collective or individual, and the type and frequency, as a way to improve ways to intervene and help to recover.

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Preparedness in Containment of Coronavirus Disease-19 in the African Continent

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ABSTRACT

The development of novel severe acute respiratory syndrome-2 (SARS-CoV-2), the causative agent of the continuous coronavirus diseases-19 (COVID-19) pandemic, has become a global concern. The current COVID-19 pandemic should be extensively comprehended to deal with it and to forestall a future pandemic. Like other countries, the African countries have also taken measures to stop the spread of COVID-19 infection like full lockdown and enforcing travel limitations. This review aimed to highlight the factors associated with the emergence, surveillance, preparedness, containment of COVID-19, along with the biosafety research facilities in African countries. A literature search with the combined keywords "Africa and COVID-19" was performed using different search engines like Pubmed, Google Scholars, and Medline Plus. The data was collected and analyzed. It has been observed that most infection spread is attributed to improper hygiene/protective measures, for example, hand washing and social distancing. Accordingly, the large scale advertisement and conduction of the COVID-19 educational programs are highly recommended. The African countries lack appropriate numbers of biosafety level 3 and 4 research facilities, trained personnel/emergency units, and funding resources to combat COVID-19 and similar pandemic. It is advisable to build up more biosafety research facilities, trained emergency response units, isolation units, and substantial funding agencies in every African country with clear rules to combat outbreaks like COVID-19. The African countries may also ask support from other countries with successful experience against COVID-19. The implementation of the suggested strategies will be helpful to African countries against COVID-19.

KEY WORDS: AFRICA, CONTAINMENT OF BIOHAZARDS, CORONAVIRUS, EPIDEMIOLOGY, PANDEMIC.

INTRODUCTION

Viral infections appearing within a given population with an increasing incidence or hovers to increase sooner rather than later are referred to as an emerging infection. This emerging infection can either be caused by an unknown or previously undetected infectious agent that has spread to new geographic areas or new populaces and whose role in the disease pathogenesis has gone unrecognized previously. Similarly, diseases that were once significant medical issues globally or in a specific country, and afterward declined drastically, yet are again turning out to be medical issues to a critical extent of the populace are referred to as re-emerging infections. The emergence and re-emergence of novel human viruses are of great concern, most notably with the emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which was confirmed to be the causative agent of

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the current coronavirus disease-19 (COVID-19) pandemic that is ravaging the world (Weber et al, 2019; Weber et al, 2019; Luo and Gao, 2020; WHO, 2020a).

It is relevant to realize that before 2003, many coronaviruses were known to cause severe infections in animals, while human coronaviruses were ordinarily connected with moderate respiratory diseases. With the constant exposure of humans to animals viruses via the food that we eat, domestic animals we rear, the animals we keep as pets, and our connections with the natural surroundings, we often get infected by several animal viruses majority of which enters and pass through our gastrointestinal tract harmlessly or get neutralized and destroyed by our competent immune system (Guan et al., 2003; Vijaykrishna et al., 2007; Jiang et al., 2017; Davis et al., 2018; Vojdani et al., 2020; WHO, 2020a).

Be that as it may, on uncommon events, an animal virus bump into a human host and starts to duplicate itself, executing its whole lifecycle inside human cells and growing one virion into a populace of many. Replication of an animal virus in the body of this first human subject is the crucial moment in the zoonotic procedure because the infection transforms and develops under the specific limitations of the human body adjusting and developing itself for replication in this new host. High viral titers generated by viral replication encourage its spread to a subsequent human host, starting choice for variations with expanded ability to spread in the human populace. As a result, several animal viruses were reported to infect humans with severe consequences such as the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003,

Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (Centers for Disease Control and Prevention, 2013), swine acute diarrhoea syndrome coronavirus (SADS-CoV) in 2016 and now, the 2019 novel human coronavirus (SARS-CoV-2), that has brought about the pandemic. Be that as it may, these rising infections are understudied in Africa yet should be extensively comprehended to deal with the present pandemic and forestall a future pandemic. This narrative aims to review the factors associated with the emergence of COVID-19, surveillance preparedness and response to pandemics, surveillance and containment at the borders, containment of COVID-19, total lockdown as a measure of containment, and biosafety laboratories in the African continent (Zhong et al., 2003; Plowright et al., 2017; Gallagher et al., 2018; Zhou et al., 2018; Warren and Sawyer, 2019; Lai et al., 2020; WHO, 2020a).

Factors Associated With the Emergence of COVID-19: There are numerous factors associated with the development of new viral diseases or the reappearance of viral infections. A portion of the factors results from standard procedures, for example, the advancements of pathogens after some time. Yet, many are an after effect of human conduct and practices, taking into account how the interaction between the human populace and our environment has changed, particularly in the last century as a result of population development, relocation from rural regions to urban communities, global air travel, destitution, and ecological destruction for economic development and land use. To establish an emerging infection, the infectious agent must enter into a vulnerable host strived and spread readily to another host, causing disease in the new host and sustain its transmission within the population. This is in line with the germ theory of disease transmission that was established by Louis Pasteur.

The expansion of human activities to new geographical areas destroying vegetation and the ecosystem, increases the chances of human contact with animals the potential zoonotic transmission of viruses. However, the genetic make-up of the host and the virus is significant in determining which animal virus will strive and replicate the first human host. This is because animal viruses required minimal mutations in other to jump species. About 70% of emerging human infections are of zoonotic origin, and two-thirds of them are acquired from wildlife. This is because unplanned urbanization leads to the destruction of the animal habitat, which exposes human contact with arthropod vectors of viral infection and animal reservoirs of viral infection. Human and animal interactions are hindering wild animals.

Most notably, in Africa, many animal species population has reduced drastically due to human activities, such as hunting, pastoralization, habitat modification, and bush burning. The significance of every one of these elements rely upon the species, its area and natural surroundings, and population density. This interaction increases human susceptibility to novel viral infection in the absence of immunity against the invading novel viruses leading a pandemic. A more extensive comprehension of how infections advance is currently being discovered by considering host hereditary components accountable for repelling infection intrusion (Pasteur et al., 1879; Broecker and Moelling, 2019; Spyrou et al., 2019; Esser et al., 2019; Tam et al., 2019; McLennan et al., 2019; Otieno et al., 2019; Ingala et al., 2019; Beena and Saikumar, 2019; Warren and Sawyer, 2019; Ramalho-Ortigao and Gubler, 2020; WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

Surveillance Preparedness and Response to Pandemics: Thoughtfulness over emerging and re-emerging infections have brought about some national, territorial, and global actions to re-establish and improve surveillance and control of transmittable infections. To strengthen surveillance, WHO member states passed a resolution urging all member states to reinforce their surveillances and capacity to detect emerging viral infection and the ability to identify novel viruses causing infectious diseases. The accomplishment of these resolutions relies upon the capacity to acquire data on viral diseases and the readiness to impart this data broadly and globally. This resolution has been made by WHO into the establishment of the Division of Emerging and other Communicable Diseases Surveillance and Control, whose duty is to reinforce national and worldwide limits in the

prevention and control of infectious diseases for a useful and timely response.

Africa, as a region, has been characterized by a higher burden of infectious diseases and has the weakest public health structure for surveillance globally, which often results in lots of paperwork, administrative bottleneck, too many instructions, conflicting priorities, and terminologies. There is a need for the use of standard case definitions, streamlined communication, strengthened surveillance, and feedback systems and training and research opportunities to improve the situation. This is the right time for Africa to move towards integrated surveillance of diseases and pandemic preparedness and response (WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

Similarly, "One Health" surveillance and emergency response should be integrated into addressing the persistent menace of emerging pandemic threats across Africa. This will offer first-hand prospects in understanding the interface of animal-human, environment, and increasing public health awareness of zoonotic diseases as well as resilience and preparedness strategies. To achieve optimal resource allocation and technical assistance, it is essential to explore emergency outbreak schemes initiative and integrated community health capacity development at all levels to alleviate the menace of future emerging outbreaks in Africa. Pandemic readiness requires exceptional degrees of political and economic related commitment. It is taxing, however realistic. The well-being of Africa, and the world, rely upon us all keeping our responsibilities (WHO, 1947; WHO, 1998; WHO, 2014; Errecaborde et al., 2019; Rivers et al., 2019; Mboussou et al., 2019; WHO, 2020 a).

Surveillance and Containment at the Borders: In several African countries, the quest for emerging viruses keeps many international researchers and their local collaborators busy. In European countries, it has motivated the enactment of preparation strategies like sorting passengers at the airport and drafted guidelines that will test health care response to the pandemic. It is in this light that some African countries recommend a technical solution to fight against emerging viruses by installing a non-contact thermometer that measures temperature remotely, without contact for screening people at the borders. The data obtained are transmitted to the situation room at the terminal to identify febrile individuals that should be quarantined at the airport to protect the public and to the health authorities for epidemiological action. This will enhance the ability to respond to the spread of infective viruses under surveillance. Nonetheless, Africa's permeable land outskirts stay a reason for worry among health authorities and policy makers, because unchecked movement and transport between countries could spread infections rapidly, as observed with the current COVID-19 hitting hard on African countries with major international airports (Bowen and Laroe 2006; Gold et al., 2019; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020; WHO 2020a).

Containment of COVID-19: In the context of the 2019 coronavirus disease (COVID-19) containment, individuals with risk are highly recommended to be quarantine. Quarantine is defined as separation or restraining of the activities of persons who are exposed to an infectious agent to monitor symptoms and for early detection of cases. Initiating early quarantine measures in an outbreak will help in delaying the introduction of diseases to a country and delay the peak of the epidemic in an environment where local transmission is ongoing. Though quarantine may generate a further source of infection if not properly implemented with regards to the current COVID-19 episode, the worldwide control system incorporates the rapid identification of lab-confirmed cases and their management or isolation at home or in a health care facility (Anderson et al., 2020; WHO, 2020a; WHO, 2020b; Coomes et al., 2020; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

A quarantine facility should be appropriately organized in such a way that it provides adequate ventilation, spacious single rooms, toilets, and hand hygiene facilities. If single rooms are not available, a large room that is adequately ventilated with beds placed two meters apart, with an appropriate level of conforming sound waste management system, is required. Sadly, these standard quarantine measures are hardly available in most of the African nations due to their low socioeconomic status. At the same time, in some countries, there are available resources, which are not adequately channelled because of displacement of priority by the government and lack of political will. At the point when a home isolate is picked, the individual ought to involve an all-around ventilated single room.

If a separate room is beyond the realm of imagination, keep up a separation of two meters from other family individuals, limiting the utilization of shared spaces and cutlery and guaranteeing that common spaces, for example, kitchen and washroom are very much ventilated. In most African countries, home or self-isolate is practically unimaginable, most notably among poor people living in the ghettos and vagrants with no professional stability. This gathering of the individual needs to go out day by day to search for what they will eat and take care of their families on a daily bases; for them, home isolation implies starvation if there is no help from the government as palliative (Sorooshian, 2020; WHO, 2020a; WHO, 2020b; WHO, 2020c).

Total Lockdown as a Measure of Containment: African countries effortlessly duplicated the format of "stay at home" or lockdown orders, as in most Western nations, however, didn't duplicate the exact circulation of monies to residents. Most African nations don't have realistic demographic data to recognize and focus on the most vulnerable points, unlike in the western world. In Africa, money is given to individuals who will end up misappropriating it and post photographs to legitimize their spending. The COVID-19 cases continue expanding each day, and if the lockdown is an approach to lessen or end the spread of the virus, at that point from the

outcomes, it's not working and counterproductive indeed as cases proceed to double. People continue to stay at home without a wellspring of income and the necessities of life. So also, for those with little reserve funds, it will soon be depleted, and individuals will be compelled to come out to hustle for sustenance (Alfani et al., 2019; Hamelin et al., 2020; WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

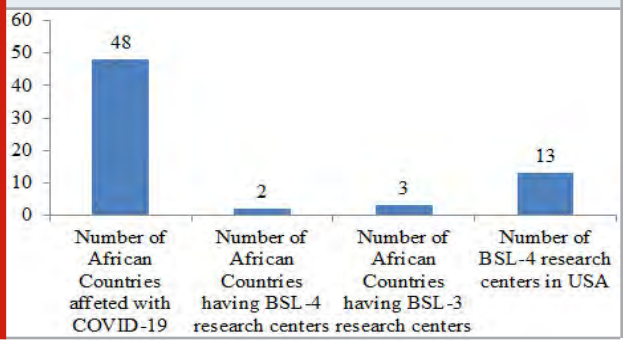
Africans have had a high degree of poverty and earning, even without COVID-19. Presently, this equitable tosses Africa into more hunger and will soon lead to individuals having more medical problems results from malnutrition and hunger. In typical circumstances, thousands pass on consistently in Africa because of different sicknesses and transmittable infections, for example, cholera, jungle fever, Lassa fever, tuberculosis, Zika infection, measles, smallpox, and so forth. The lockdown intensified these ailments as most of the casualties now have almost no money to take care of themselves. The common man considers this as an elite problem because, to them, the "hunger Virus" is more terrifying than SARS-CoV-2. The more significant part of the proprietor of micro, small and medium enterprises are probably going to devour their business capital during the lockdowns, with no reasonable helpline. This is because the palliative measure taking by some African countries won't almost certainly arrive at the targeted population because of dishonesty and fraud. Horticulture in Africa depends on downpour and season. The lockdowns during the planting season could undermine food security. This will have adverse effects on the farmers and the consumers, along with the increased inflation (Alfani et al., 2019; Hamelin et al., 2020; WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

Biosafety Laboratories in African: At the point when we discuss containment laboratory, two things ring a bell; biosafety and biosecurity. Biosafety is a containment guideline, expertise, and practice that is executed to forestall unexpected exposure to biological agents and poisons or their negligent discharge. At the same time, biosecurity is the protection, control of organic agents and poisons inside the research lab, to forestall their misfortune, abuse, re-routing, unapproved access, or purposeful unapproved discharge. All tasks performed in bio-labs are classified by their biosafety levels. Biosafety levels (BSL) are utilized to recognize the protective measures required in a lab setting to ensure the protection of employees, the surrounding environment, and the general public. There are four biosafety levels. These are BSL-1, BSL-2, BSL-3, and BSL-4, with BSL-4 being the maximum containment level of all the BSL (Gronvall and Bouri, 2008; Gómez-Tatay and Hernández-Andreu, 2019; Iwen et al., 2020; WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

Level 4 BSL is used for studying high-risk infectious agents that are capable of aerosol transmission and life-threatening infection with no available vaccine-like SARS-CoV-2 causing the current COVID-19 pandemic. In Africa, there are just two BSL-4 research centers situated in Gabon and South Africa, and only three African nations have standard BSL-3 labs situated in Nigeria, Kenya, and South Africa (Figure 1). Contrary to this, the United States has 13 operational BSL-4 labs (Bressler and Hawley, 2006; Ahmad et al., 2015; Carpenter and Bhadelia, 2019; WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

Figure 1: Standard biosafety, level 3, and level 4 laboratories in the African continent

Figure 2: Number of BSL centers in Africa and the USA



It is because of the inadequacies of BSL 3 and 4 in Africa that stimulate WHO to come of African Emerging and Dangerous Pathogen lab Network (AFR-EDPLN) to provide diagnostic services for an array of emerging pathogens. The objective of the AFR-EDPLN is to improve readiness and response to Emerging and Dangerous Pathogens (EDP) by upgrading diagnostic abilities and giving better access to a scope of tests for EDP, encouraging increasingly quick reaction and improved outbreak control process. The EDP system, as of now, involved 14 national consisting of Uganda, South Africa, Sierra Leone, Senegal, Nigeria, Madagascar, Kenya, Ghana, Gabon, Democratic Republic of Congo, Côte d'Ivoire, Central African Republic, Cameroon, and

Algeria. In any case, there is a requirement for a powerful, dependable and versatile system of research centers with the ability to distinguish emerging and re-emerging infections so that Africa is more ready to identify and react to future vulnerabilities to regional health security and reinforce the African ability to contain emerging and re-emerging infections (Boeras et al., 2016; WHO, 2017; Balajee et al., 2016; WHO, 2020a; Martinez-Alvarez et al., 2020; Chinazzi et al., 2020).

Most infections spread is attributable to improper hygiene and protective measures, for example, hand-washing, cleaning, and safe entombment practices. It is, therefore, critical to keeping up essential cleanliness and protective conduct, for example, social distancing. With fewer Biosafety Level 3 and 4 research facilities in African countries, it is essential to build up more labs in Africa or increases the number of regional labs. It will likewise be astute to set up an emergency response team or unit in every African country, having all the vital training and transparent rules for handling emergencies, which can act following an outbreak in collaboration with regional BSL-4 lab. African scientists and clinicians should be provided with training opportunities on biosafety and biosecurity as well as the standard containment principle in managing pandemic.

There is also a need to establish isolation units in specialized hospitals, along with the necessary infrastructure and protocols to monitor infected patients. There should be substantial funding allocated by governmental and non-governmental organizations to improve primary health care infrastructure in African countries to ensure that emergency medical situations are appropriately tackled. The large scale advertisement and education are also highly recommended. Finally, the African countries may also ask support from other countries with successful experience against COVID-19. The implementation of the suggested strategies will be helpful to African countries against COVID-19.

CONCLUSION

In conclusion, the large scale advertisement and conduction of the COVID-19 educational programs are highly recommended in African countries. They lack appropriate numbers of biosafety level 3 and 4 research facilities, trained personnel/emergency units, and funding resources to combat COVID-19 and similar pandemic. It is advisable to build up more biosafety research facilities, trained emergency response units, isolation units, and substantial funding agencies in every African country with clear rules to combat outbreaks like COVID-19.

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Arabidopsis thaliana a Medicinal Plant As a Genetic Model System in Crop Science and Scientific Fields

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ABSTRACT

Arabidopsis thaliana is the most popular plants in scientific fields whereas it is used as molecular genetics plant model because it can be manipulated easily and has a relatively small genome of approximately135 mega base pairs. This plant possessed many known mechanisms of DNA repair, producing an unusual pattern of inheritance. Because of the unique characteristics of the plant, *A. thaliana* has considered as a model plants for many studies. It is also extensively studied as a model for flower development. Importantly, the integral sequence of the Arabidopsis genome allowed the swift discovery of the molecular basis of recognizing mutant plant, which made it convenient and a powerful to recognize genes that are involved in many aspects of the plant life cycle. This review deals with the unique importance and characteristics that make *A. thaliana* a better choice for scientists and researchers interested in the study of living things, particularly those with interest in molecular plants.

KEY WORDS: ARABIDOPSIS THALIANA, GENE EXPRESSION, POLLEN, ANTHER, MUTANTS.

INTRODUCTION

Although the *Arabidopsis Thaliana* is currently found in different parts of the world, the plant is thought to have existed in Europe and Asia at first. As biologists and other scientists developed an interest in *Arabidopsis Thaliana* plant, *Arabidopsis thaliana* went through several stages under which it was given different and unique names. At some point, the plant was identified as Pilosella siliquata by a biologist called Johannes before the name changed to *Conringia thaliana*, *Pilosella thaliana*, and *Sisymbrium thalianum*. Much later, the name was changed to *Arabidopsis thaliana* a code name that inferred the plants usefulness in the study of genes (Provart et al., 2016, Yang et al., 2017, James 2018, Neetu 2019). After decades of research, studies revealed that *Arabidopsis thaliana* was a perfect model organism that could be used in the study of genes. As a model organism, *Arabidopsis thaliana* has crucial characteristics. It takes a short duration time to grow, tolerate high temperatures, need no fertilization and it can achieve the same growth milestones when planted indoors. Moreover, it has small seeds and genome of 132 Mbp and grows perfectly under tough conditions such as under turbulent wind exposures.

The Genome Sequence of *Arabidopsis Thaliana*: The *Arabidopsis thaliana* genome is often used as a model for other plants following a series of studies and apparently, *Arabidopsis thaliana* was reliable for genome sequencing. Out of the required 125 megabases that define genome regions, the *Arabidopsis thalianaha*s more than 115.4 megabases (Quilichini et al., 2015). Nonetheless, the plant has numerous types of proteins with diversity which

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making up the plant as the best choice for hybridization and crop development, (James 2018, Dennis 2018, Neetu 2019).

Figure 1: The life history of the *Arabidopsis thaliana* from seed to maturity after 50 days (Boyes et al., 2001).

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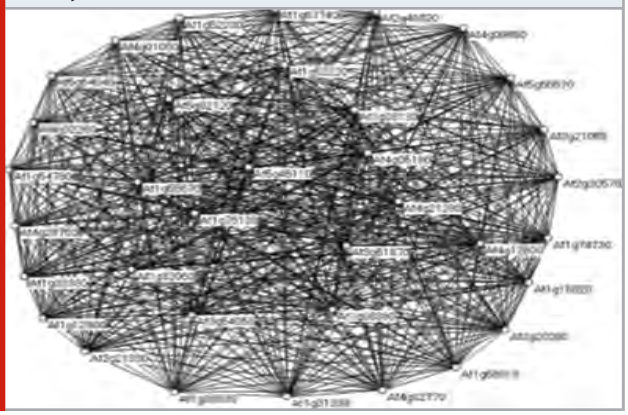
Figure 2: *Arabidopsis thaliana* genes (Yamada, et al. 2003).



Effect of 5'UTR Introns on Gene Expression in ***Arabidopsis thaliana***: Intron refers to the nucleotide sequence found in genes, which is removed by RNA splicing at the maturation of the final RNA product, was first discovered in 1977 (Achard et al., 2004). The two researchers and scientists came up with the name introns rather than exons that code for gene products. According to subsequent studies, particularly plant expression studies in chimeric RNA, the intron sequence can reinforce the level of protein expression, a phenomenon called Intron-Medicated Enhancement (Callis et al., 1987). Thus, introns within the 5'UTR exhibit specific features that make them different from the introns found within the coding sequence and the 3'UTR. Pertaining to the EF1α-A3 gene, the presence of a long intron in the 5'UTR is sufficient to enhance gene expression in plants.

The *Arabidopsis thaliana* Chloroplast Proteome and Protein Functions: In photosynthesis, the process through which plants manufacture food, chloroplast proteome organelles pick up energy from sunlight, convert, and store it in energy storage molecules while releasing oxygen from the plant and algal cells. Chloroplasts are of cyanobacterial origin although their autonomy during evolution and development (Anthony and Frank, 2005). They usually transfer part of their genes to the nucleus. Apparently, part of the genetic information found on proteome constitutes the chloroplast and the metabolic functions that define the protein complement of *Arabidopsis thaliana* plastids.

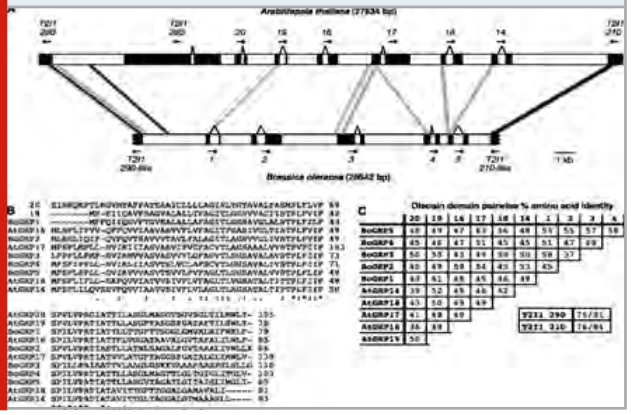
Linking Genes with Ecological Strategies in *Arabidopsis thaliana*: From the oncological perspective, *Arabidopsis thaliana* exhibits a collection of numerous diverse genotypes defined by a complex population structure. Its phenotypic variation makes it a dependable plant for the study of genes. Importantly, it is applicable in studies because it has high-adaptation capabilities when subjected to varying environmental conditions (Callis et al., 1987). Owing to the phenotypic change capacity, *Arabidopsis thaliana* copes with environmental differences as may be necessary when conducting studies (Anthony and Frank, 2005). Co-variations between the plant traits are a replication of evolution, trait syndromes, and are a revelation of biodiversity.

Protein Oxidation in *Arabidopsis thaliana*: In *Arabidopsis thaliana*, protein oxidation is an irreversible procedure that involves the modification of side chains in a few native amino acids such as histidine, cysteine, and lysine. Often, the oxidation process results increase in the levels of carbonyl and a dysfunctional protein during the first few days of the *Arabidopsis thaliana* life cycle (Provart et al., 2016). Particularly, experiences a significant reduction in protein carbonyls prior to bolting and flower growth over the first 20 days after germination (Becker et al., 2003). However, the model of the plant is best utilized if kept under optimal growth conditions during the first 20 days. After fertilization, and as the seed matures, the plant's somatic tissues usually become more senescent. Apparently, leaf senescence defines the last step of leave development considered as a vital step towards the death of the plant's life cycle. With consideration of the carbonylation of *Arabidopsis thaliana* during

the various stages of the plant life cycle, it is clear that the plant is a unique life form to consider and use in scientific studies.

Gene Families Defining Arabidopsis thaliana Pollen Coat Proteome: *Arabidopsis thaliana* pollens are comprised of protein intermediate species and composition needed for effective pollination. The pollens include over 10 kilo Daltons of proteins and genomic clusters that correspond with genes (Birnbaum et al., 2003). With lipids, the proteins are utilized by flowering plants much as they are coated with sophisticated extra-cellular pollens. These interact selectively with receptive female stigma cells. Apparently, the coating makes it possible for plants with dry stigmas to connect since they have a functionally similar lipid-rich exudate on the surface of stigmas. Previous studies have revealed significant evidence of synteny between *Arabidopsis thaliana* and *Brassica oleracea* clusters as observed from the comparison of oleosin clusters and flanking DNA. These differences are observed in black boxes, exons, arrows, and the direction of transcription, regions of BLASTN value of E, and the black connecting bars (Birnbaum et al., 2003). Additional aspects that show variations include the grey connecting bars and regions of Value E at 10220 as well as the oleosin domains.

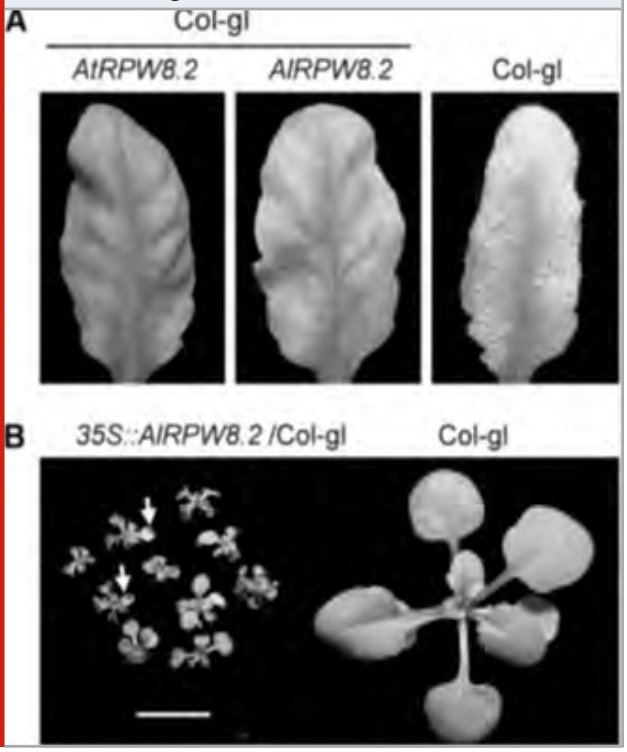
Figure 3: Intraspecific Genetic Variations, Fitness Cost and Benefit of RPW8, A Disease Resistance Locus in Arabidopsis thaliana (Orgil et al., 2007).



Arabidopsis thaliana features a unique RPW8-encoding class of genes with its origin in ancient land plants. Over the years, the genes evolved through processes such as domain fission, fusion, and duplication to confer the resistance needed counter pathogens such as powdery mildew. Essentially, two homologous genes, particularly, RPW8-1 and RPDW8-2 best illustrated in the following images, define this locus.

In the experiment, a 2.3 kb genomic fragment with AIRW8-2 and its promoter were introduced in Col-gl background with a transgenic plant inoculated with cichor-acearum USCS1. Additionally, disease phenotypes were recorded and typically infected leaves photographed after 10 days of exposure in post-inoculation. Notably, the researchers expressed the AIRPW8-2 gene under the 35S promoter and Co-gl background. It emerged that plants with up to 10% transgenic lines exhibited SHL whereas the 4-week-old T3 plants showed a single line (Achard et al., 2004). The most severe SHL was seen in the wild type. Arrows were interpreted as signs of dead or dying leaves. Speculatively, the mutation would appear at frame shift and truncation of up to 34 amino acids at the C-termini or reduced proteins (Hudson and Kaplan, 1985).

Figure 4: AlRPW8-1 and AlRPW8-2 (Hudson and Kaplan, 1985, Xiu-Tang et al., 2012).



Regulation of the developmental processes of ***Arabidopsis thaliana:*** Plant growth and development is depended on a wide range of genetic factors that trigger the production of the hormones needed for. Arabidopsis thaliana stands out as an important plant for biological studies because the plant exhibits variable hormones such as the gibberellin (GA) that coordinates the development process of different plant parts (Yang et al., 2017). Ordinarily, plants grow under abiotic stress that they must cope with for survival. To cope with the stress, plants require morphological adaptation capabilities that are primarily facilitated by hormones (Smalle et al., 1997). For instance, plant hormones regulate and mediate the morphological response of roots under adverse soil conditions. Particularly, plants rely on jasmonic acid for mediation of stress response among other developmental processes, such as metabolism and biosynthesis (Stefanie et al., 2015). In *Arabidopsis thaliana*, the auxin hormone regulates the development of seeds, a process that involves cotyledon formation, pollen grains formation, and hypocotyl cell elongation among other key processes. Hence, *Arabidopsis thaliana* is considered an important model to use in biological studies.

Pertaining to the maintenance of a unique R gene locus, there are two key observations to note. Firstly, genetic variation is noted in high levels of RPW8, a sign of zero selective sweeps for the locus. Secondly, benefits and costs are observed in the RPW8 expression although depending on the fitness of individual plants, which is in turn influenced by exposures to pathogens as well as the development of anthers (Baesso et al., 2018). This is in view that the Arabidopsis LFR Gene is a crucial requirement in the formation process of Anther Cell Layers. As usual with any other process, genes play a key role in anther development; the molecular technique behind the transcriptional regulation of associated genes is indefinite (Zoe et al., 2011). The leaf and flower relationship are deducible, which is a reaffirmation that all genes are a crucial component of the genetic network that modulates all the plant processes.

In *Arabidopsis thaliana*, the receptor-like protein kinase-2 (RPK-2) plays an important role in the control of anther development. Ordinarily, the receptor-like kinases (RLK) falls in a large gene family within the Arabidopsis genome and plays a central role in plant growth and development. It is an important determinant of plant response to hormonal changes and stress, which makes it a key regulator of anther development in Arabidopsis (Becker et al., 2003). Ostensibly, defects in anther dehiscence and pollen maturation trigger enhanced shoot growth and male sterility. Rpk-2 anthers, which are one of the primary insertional mutants, tend to develop into three cell layers around the male gametophyte (Cecchetti et al., 2008). The middle layer is not necessarily differentiated from inner parietal cells.

The pollen mother cells can often afford meiosis although subsequent differentiation might be inhibited by tapetum hypertrophy. The resultant pollen grains tend to exhibit aggregated morphologies. Besides, the presence of microspores and tetradson anthers, as might be noticed during microspore formation, are a sign of developmental homeostasis (Claudia et al., 2004). Often, anther locules crush without necessarily undergoing stomium breakage, a phenomenon largely presumed to be a consequence of lignification and inadequate thickening of the endothelium (Birnbaum et al. 2003). Based on results of microarray analysis, most genes encoding metabolic enzymes, such as those involved in metabolic processes on the cell walls and lignin biosynthesis, tend to downgrade throughout the anther development process. Therefore, RPK-2 controls the tapel cell fate by invoking subsequent tapetum degradation. Mutating RPK-2 impairs pollen maturation as well as the anther dehiscence, which is a result of disruptions on key metabolic processes.

GAMYB-like genes, particularly MYB33 and MYB65, as they are found in Arabidopsis are microRNA-regulated genes that facilitate anther development redundantly. Previous studies have not revealed any significant information about the gene encoding for R2R3 MYB domain proteins as found in Arabidopsis (Achard et al., 2004). However, closely related genes MYB33 and MYB65 found in Arabidopsis thaliana have been shown to have great sequence similarity with the GAMYB gene found in barley, (Haseneyer et al., 2008). In an earlier study where T-DNA insertional mutants were isolated, findings showed that a myb33 and myb65 double mutant was defective in anther development. Ostensibly, it emerged that tapetum undergoes hypertrophy within the cell stage of the pollens, which results in a pre-meiotic abortion of the pollen development process. However, sterility is conditional in that fertility increases under higher light conditions as well as when Arabidopsis thalianais placed in lower temperature conditions (Zoe et al., 2011). MYB33 and MYB65, therefore, are not necessarily essential for the development of anthers although the genes seem to facilitate the process.

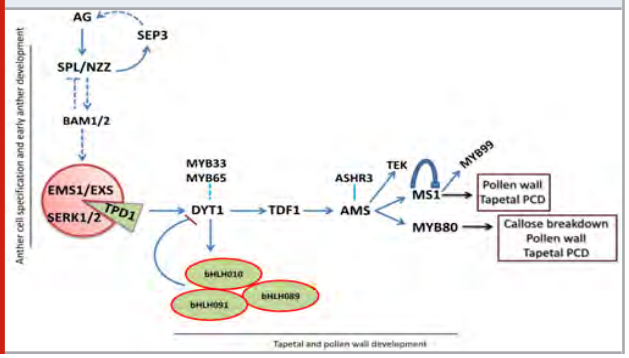
In a manner consistent with functional redundancy, promoter–β-glucuronidase (GUS) fusions of MYB33 and MYB65 give identical expression patterns in flowers. The impact is noticeable in kin sepals, receptacle, and anther filaments (Claudia et al., 2004). It is not necessarily limited to anthers although it is traceable in shoot apices and root tips as well. In relatively young anthers, the expressions of MYB33 genes is consistent with the male-sterile phenotype and no staining as a result of shoot meristems. From a micro-RNA sequence, however, the fusion between MYB33 and GUS results in an expansive expression pattern and tissues similar to the promoter-GUS lines. The interpretation is that the micro-RNA target sequence is a restriction of MYB33 (Birnbaum et al. 2003). Ostensibly, Arabidopsis infused with MYB33 and mutated mico-RNA results in dramatic pleiotropic developmental defects. A restriction of MYB33 in shoot apices, therefore, is highly recommended for the proper development of Arabidopsis thaliana.

The Regulation of Anther Development in ***Arabidopsis***: In Arabidopsis, anther development involves several steps, particularly, 14 stages that culminate in histogenesis. Neetu (2019) reports that anther development in Arabidopsis is characterized by a complex network of transcription factors that define the 14 stages. Importantly, molecular knowledge of the anther development process is crucial for a clear understanding of the stamen control, which entails the manipulation and study of male fertility. Exposition of the composition and structure of the filament, as well as the anther that produces or contains pollen grains, is tantamount when studying *Arabidopsis thaliana* as the biological model for the study of genes (Neetu, 2019). Essentially, all Arabidopsis anther genes can be combined into simple pair genes and co-expressed networks to construct a co-expression network. The result is a combination of 254Arabidopsis anther groups. Considering that the combination of co-expressed groups contains a high propensity of functionally related and co-existent genes, it emerges that *Arabidopsis thaliana* is a uniquely important model that could open up the field of biological research.

At the transcriptional level, regulation of gene expression helps to control a significant deal of cell physiology in order to manipulate and mediate the development of

tissues. Apparently, the development depends on the differential gene expression in all the cells involved in the differentiation and specification of organs (Neetu, 2019). The process is controlled by transcriptional factors that act as regulatory switches. Importantly, the 14 stages that define the anther development stages are categorized into two key phases: microsporogenesis and microgametogenesis. Stages one through to seven make up the microsporogenesis phase and involve the differentiation as well as the meiotic division of microspore cells. The stages from eight to fourteen make up the microgametogenesis phase in which microspores are emitted from tetrads before the mitotic division of the same microspores, the process that results in the production of pollen grains. Degeneration, which is crucial for the maturation of pollen grains and disposal, occurs as the last stage (Neetu, 2019). The following diagram showed the entire process in a nutshell.

Figure 5: Regulatory network of transcription factors of anther development (Juanying et al 2016). the diagram, arrows represent positive regulation whereas the T bar represents negative regulation. Dashed lines represent the putative regulatory function.

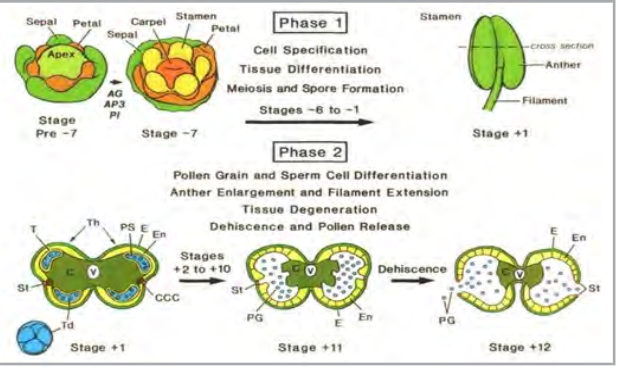


In the early stages of anther development, AG acts as the primary trigger where NZZ/SPL acts in a positive loop relative to the AG. The NZZ/SPL up regulates both the BAM1 and BAM2 expression, in a negative loop expression. Notably, EXS/EMS1 forms a receptor complex that includes SERK1, SERK2, and TPD1 that binds to the receptor complex. In the process, tapetal development is activated by TDF expression that triggers DYT through the up regulation of AMS expression (Smalle et al., 1997). In return, AMS up regulates the expression of MS1 and MYB80 that contributes to the development of pollen walls.

Basic Principles and Applications of Anther Development: The development of anthers occurs in stamen since anther constitutes the male reproductive part of flowers. Also called the sporophytic system, the stamen contains diploid cells that usually undergo meiosis to produce haploid male spores or microspores. These microspores usually divide and differentiate into multicellular gametophytes or pollen grains. As the male part of the flower, the stamen is morphologically distinct and unique component supported by the filament, which is a vascular tissue that serves as the nutrients and water channel (Becker et al., 2003). Broadly speaking, the anther development process occurs in two phases.

The anther morphology is established in phase one where the cell and tissue differentiation occurs with the microspore mother cell undergoing meiosis. By the end of the first phase, the anther should contain almost all the specialized cells and tissues. Tetrads of microspores are usually evident in the pollen sacs. The second phase is characterized by the differentiation of pollen grains. The anther enlarges and advances upwards within the flower through a process called filament extension. Eventually, pollen grains are released (Miransari and Donald, 2016). Through regulation of cellular processes within the flower, differentiation of anther cells occurs with the establishment of tissue patterns. The anther switches from the histo-specification programs of phase one to the cell degeneration and dehiscence program of the second phase (Cecchetti et al., 2008). The developmental events leading to anther formation and the release of pollen grains are exquisitely time (Robert et al., 1993).

Figure 6: An overview of anther development phases and stages (Cecchetti et al., 2008).



Similar to any other developmental process involving cells, anther development is equally subject to defects, particularly, defined as Arabidopsis thaliana male-sterile mutants. Within the stamen, primordia cell-specifications and differentiations appear early and mature faster than other cell types. They generate strange anther morphology from what is expected in ordinary flowering plants. Clearly, defects in anther development result in futile phenotypes and are largely associated with sterility mutant screens (Robert et al., 1993). posits that sterile mutations are a common occurrence in many of the known flowering plants. The mutants range from anther morphology to micro- sporogenesis, pollen dysfunctions, and under-developments.

Protein Phosphorylation for the Regulation of Anther Development in *Arabidopsis*: The development of stamen, which is the male reproduction part of the flower, is a sophisticated process that involves primordium initiation and early cell divisions. Other crucial process includes differentiation, generation of haploid microspores, and the formation filaments (Smalle et al., 1997). Ordinarily, a mature anther should have four lobes where each lobe contains microspore cells and somatic tissues.

The tissues should also contain four sporophytic cell layers independently, epidermis, endothecium, and tapetum. In the first phase defined by the seven stages, archsporial cells undergo differentiation and division to form the building blocks if the stamen. In subsequent stages that define the second phase, anthers are formed and pushed upwards into their position. Additionally, sperm cells are formed as pollen grains mature up. Numerous regulatory factors, however, come into play throughout the two phases (Robert et al., 1993). These factors signify the significance of *Arabidopsis thaliana* as an important model of study in modern genetic and biomedical research.

Pollen Development and *Arabidopsis*: The development of heterotrophic pollen grains usually requires energy supplementation with carbon inputs. In the early stages, spores are immersed in a mixture of locular fluid and nutrients from sporophytic petal cells. During the course of pollen maturation, a buildup of carbohydrates with varieties of lipids and starch is paramount (Sangeeta and Maria, 2008). Essentially, nutrient filling throughout all these stages is essential for eminent fertilization. The growth of the plant structure during pollen germination occurs in the early stages although it is manifested a little later in some cases (Robert et al., 1993). Notably, induced male sterility, as is usually attributed to the shortage of carbon or energy sources can be achieved at this stage. As a fact, thorough knowledge of the nutrient regulation process throughout the pollen maturation process is important in agricultural practice.

Regarding *Arabidopsis*, the maturation of pollens involves the action of lipoid bodies although starch comes as a gift during the initial stages of pollen development. The formation process of lipoid biogenesis bodies, as found in pollens, is analogous or similar to the formation of oil storage bodies in oilseeds. In both cases, the formation process involves two key steps that occur in different organelles. Essentially, the initial step called the Delaware nivo stage involves the synthesis of fatty acids into acyl CoA and carbon. The second step, however, is unique in that it occurs in the specialized endoplasmic reticulum (ER) cells where acyl-CoA mixes with glycerol-3-phosphate (G3P) to produce triglycerides (Sangeeta and Maria, 2008). As an important point to note, carboxylic acids in pollens usually undergo synthesis in non-photosynthetic plastids, a process that depends on the importation of carbon. The carbon is derived from a variety of sugars and glucose-6-phosphate (Glc6P) that is converted into plastids. Unfortunately, existing literature does not document enough information on how the various steps are coordinated to an exceedingly spatiotemporal-specific manner. Besides, it is unclear which step involves rate-limiting for the lipoid body biogenesis process.

Various common proteins are known to exist in the pollen coat of Arabidopsis and maize. Apparently, these proteins have been shown to play a crucial role in pollen-stigma interactions during fertilization in both cases. An example of these proteins is the oleosin-domain supermolecule GRP17 where the enzylem EXL4 genus are from the genus Arabidopsis pollens. Besides being coated, these proteins coordinate the association between pollens and the stigma. Pertaining to maize, however, xylanase is discharged from the pollen coat during the initial stages of fertilization. Importantly, the discharge facilitates the penetration into the silk though the aid of xylan chemical that acts as the catalyst (Sangeeta and Maria, 2008). In both the maize and *Arabidopsis* cases, molecules usually settle at the surface of pollen grains, which is vital for the interaction of the grains and the stigma during the pollination process.

While acknowledging the mode of interaction between pollens and the sigma, as noted in the case of maize and Arabidopsis, it is important to note that there is only limited literature documenting the role of signals from within pollens during self-pollination process. It was only until recently when the genus *Arabidopsis* mutant that is associated with nursing came to the limelight among researchers (Harry, 2004). Presumably, the genus is considered to be a possible cause and reason for impaired pollination in vivo where it acts by reducing jasmonic acid levels in pollen grains. Biologists and other researchers postulate that aberrant peroxisome morphology is a possible regulator and controller of peroxisome biogenesis found in pollens. Notably, aberrant peroxisome morphology is expressed in the vegetative cells of pollen grains (Harry, 2004). The interpretation is that the jasmonate acid signals received from within pollen grains usually act as the regulators of the germination process and the interaction with stigma in *Arabidopsis*.

Tapetal Expression in BnaC.MAGL8.a and its Impact on Male Sterility in *Arabidopsis:* Monoacylglycerol enzyme (MAGL) is usually used to hydrolyze monoacylglycerol, a process that yields carboxylic acid and alcohol. This protein is known to play a crucial role in the growth and development of vertebrates, its functions in plants remains a relatively unexplored area of study that requires further research (Dennis, 2018). Existing literature shows that MAGL genes often reveals a tapetal expression of BnaC.MAGL8.a, which is a homolog of AtMAGL8. Often, most attempts to combine the two results in male sterility of *Arabidopsis thaliana* or unintelligent tapetal PCD and defective spore wall in transgenic plants. Besides, tapetal cells can also develop into becoming cavum before degeneration in the last stages of development. Nonetheless, where microspores tend to degenerate with tapetal cells, few pollen grains emerge with an irregularly shaped exinelayer in transgenic plants (Dennis, 2018).

Variations in gene expressions have been explained with the argument that they are potentially a result of the response to stress among other factors that appear to threaten their growth and development.In experiments where microspores are terminated or aborted, the downstream wall biogenesis genes of pollen grains are usually down-graded. Notably, the genes linked to the reactive atomic number eight species exhibit significantly higher stability and equilibrium as their jasmonate

signals are upregulated in transgenic plants (Baesso et al., 2018). Ordinarily, these observations are interpreted as being the result of the expression of BnaC.MAGL8.a in tapetum, which invokes stress response leading to the impairment of pollen development.In the United States, the apparent similarity between atgpat1 mutant and the BnA9:BnaC.MAGL8.a from transgenic plants led to the proposition that monoacylglycerol (MAG) played a crucial role in the development of pollens in genus *Arabidopsis* (Delker et al., 2006).

The Pathway to Pollen Development Based on the *Arabidopsis*-Rice Relationship: The development of spores that discharge at the right stage of the pollination and fertilization process is paramount for a given plant species to replicate itself without necessarily compromising its genetic pattern. According to earlier transcriptomic experiments on dilleniid dicot genus, it emerged that staged spores had up to thirteen sets of 977 genes expressed within the male reproductive structure. Besides, up to fifteen genes were noted in the male flora (Delker et al., 2006). A critical analysis of these findings reveals that the scope of organic phenomena throughout the reproductive structure and spore development is replicated in genes.

The ability to control the development of spores is an important breakthrough towards effective selective breeding. Particularly, the process is actualized through the discharge of genetically charged spores; hence, the initiation of hybrid lines in accordance with given expectations (Delker et al., 2006). Oftentimes, hybrids exhibit heterosis and hybrid vigor, which implies that that the new generation of plants could have relatively stronger tissues compared to the parents. Besides, hybrid species tend to mature earlier than usual, or even give higher yields depending on the nature of gene hybridization performed. Although researchers and scientists have a wide scope of generating hybrids, it has been observed that cytoplasmic male sterile (CMS) is commonly used for most crop species such as rice, maize, and cotton (James, 2018).

The CMS approach, depending on the interest or desires of biologists, used in a couple of plant species because it triggers nuclear-mitochondrial interactions. Nonetheless, the process may result from cross-hybridization where it is naturally aided by wind or insect. Based on previous studies, however, researchers have proved that some of the CMS lines have characteristically unique features from those of the parent species. Apparently, arising defects are aberrantly associated with open reading frames (ORFs) in mitochondrial genomes. Nevertheless, some of these mitochondrial defects are recovered through the nuclear encoding of genes, a process that helps to restore the plant fertility in full. Thus, it is possible to manage or reinstate the fertility of species through the selection of the appropriate breeding lines.

Multiple transgenic approaches are available for the conjointly use in the development of hybrid seeds. Scientists have previously demonstrated a great preference for the CMS approach when developing hybrid oilseeds. The technology, as it is used for commercial purposes, is predictable for productive structure needed when specific gene expression is desired. Essentially, available alternative approaches largely rely upon barnase as a combination of two inactive peptides and ulterior reconstitutions. Many of the alternative approaches, however, involve the expression of non-functional barnase and acetolactate synthase. The idea is to achieve male sterility through ulterior interaction and inteine-based splicing of supermolecule fragments. Ostensibly, many of the modern systems are based on suppression and restoration of key genes related to spores development where common examples include MYB103. Unfortunately, these approaches come with the limitation that they are restricted by the inadequacies of existing technologies and limited understanding of their mode of operation.

The Essence of MED30 subunit of mediator complex needed for plant development: The mediator is a giant multi-protein that is considered essential for the transcription of almost all genes transcribed by the polymer enzyme II. Importantly, specific subunits are required to assemble a working, rather, useful intermediator in vitro. Thus, a corresponding loss of function mutants could result in deadly outcomes (Zoe and Da-Bing, 2009). The MED30 subunit is particularly essential in anomal systems although it is absent in yeast. The subunit is reportedly for the survival of all male plants as well as for the development of embryos. In an earlier study, for instance, researchers observed that MED30 spore grains were viable. A few germinated and targeted the ovules much as the embryos aborted after a short period of fertilization. Based on these observations, it was deduced that MED30 is important for paternal management especially during the early stages of embryo development.

DEX1 is required for Exine pattern formation during pollen formation: DEDX1 is a novel plant protein needed in the process of pollen formation in Arabidopsis. To identify the factors and conditions needed for the formation of spore walls, it is necessary to characterize the T-DNA-tagged DEX1 mutations of the Arabidopsis genes that end up having defective spore walls because of irregular formation. This assertion is supported by earlier studies that showed DEX1 mutations encode unique macromolecules that are expected to be membrane-associated but contain numerous calcium-binding domains. In the study, the researches sought to isolate and determine the molecular characteristics of the DEX1 morphological and ultra- structural patterns in plants.

Importantly, it has been noted that the development of the spore wall in DEX1 cells is similar or comparable to many of the existing wild-type plants until the first quaternion stage of development. This observation is consistent with the fact that primexine deposition is ordinarily delayed and significantly reduced in DEX1 plants (Delker, 2006). The natural riffle of the cell membrane in DEX1 and the spacers ascertained in wild-type plants is absent

in mutants. Sporopollenin is produced and deposited indiscriminately on the cell membrane in all DEX1 plants. Nonetheless, sporopollenin is not anchored on the spores (Anthony and Frank, 2005). Rather, it forms massive aggregates on the microspores as well as on the bodily cavity walls. As they are supported by the DEX1 structure and the nutrition of the plants, many of the roles of the macromolecules are certainly planned.

Pollen Development in *Arabidopsis*: The basic qualities of the gametophyte have been studied and documented in detail in previous studies although, in some cases, the qualities used to calibrate the procedures have been challenged for being seemingly obscure. Important concepts of study have been the characteristics of an obscure Arabidopsis and the GTP-Restricting protein-related 1 (GPR1). Apparently, the GPR1is communicated explicitly in ovule, dust, and in the dust tube (Zoe and Da-Bing, 2009). Additionally, upgraded green fluorescent protein-labeled GPR1 restricts both the core and cytoplasm. It also introduces punctate and ring-like structures. GPR1 is known to freak with no deformities in gametogenesis or even seed setting. The dust grains are usually pale shading on a quick glance. On examination under the electron microscope, ordinary designed and slender exines are noted on GPR1 pollen surfaces, which explain the shading pale appearance.

Researchers have also been interested in whether the GPRI transformations have a significant influence on post gametogenesis procedures, such as dust germination, ovule senescence, and dust tube development. Study findings show that GPR1 dust grains tend to sprout fast and their pollen tubes lengthen t a relatively fast pace. Notably, dust grains and ovules from GPR1 freaks show significantly low feasibility compared to the wild type when gauged based on a span of 4-5 days. These observations lead to the inference that the GPR1 capacities, as an inhibiting controller of dust germination, gametophyte senescence, and dust tube development, tend to tweak the preparation process.

Studies dedicated to the exploration of Microsporogenesis have played a key role in the analysis of the wild *Arabidopsis thaliana* as well as the atomic male-clean mutation BM3, a process that largely involves cytochemical recoloring. The freaks require adenine phosphoribosyl transferase compound made up of purine rescue pathway. Under adenine, the compound changes to AMP. Biologists observe that dust starts to veer off the wild kind soon after meiosis while the quadruplicates of microspores discharge from their callose dividers. The main sign of unusual advancement of dust in the mutant is a dark coloring attributed to the microspore divider. It is actually caused by inadequate intine blend. Under these circumstances, freak microspores do not respond to mitotic divisions. These observations amount to the reassertion that there are evidently conceivable roles of adenine rescue in dust improvements.

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Assessment of Water Quality in Some Wells of Albaha Region and its Surrounding Area, Saudi Arabia

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ABSTRACT

Aim This study aimed at assessment of some physical, chemical, and bacteriological parameters of some wells in Albaha region and its surrounding area, Saudi Arabia. **Methodology:** Physical parameters (total dissolved solids & turbidity) were analyzed by standard conductivity turbid meter. Chemical parameters (metal ions, sulfate, nitrate, nitrite and acidity) were measured by standard spectrophotometer and pH scale. EC blue 100® screening medium was employed to test for coliform bacteria in wells water. **Results:** Up to 73.7% of samples exceeded the permissible limits set by Saudi standards specified for total dissolved solids, turbidity, pH, Mn, SO_4 and NO_3.The average pH of targeted wells is 7.98 ± 0.37 which is within the permissible range specified by national and global standards. Detected levels (mg l^{-1}) of total dissolved solids, Fe, Mn, SO_4, NO_2, and NO_3 were 345.04, 0.62, 0.13, 5.74, 119.90, 52.74, and 0.157 mg l^{-1} respectively. Mn and NO_3 levels exceeded the permissible limits of Saudi and global health standards. About 36.8% of wells had a positive reaction for coliform presence by EC blue screening medium. In terms of spatial variations, no significant difference between individual sites was observed, however, sites as groups show remarkable variations in one group (SA) which had minor increases in NO_2 levels compared to other sites. Correlations between SO_4 & total dissolved solids, Mn & NO_3, pH & total dissolved solids, SO_4 & NO_3, and SO_4 & Mn levels were found. **Interpretation**: Elevated levels of studied parameters in groundwater may be linked to agricultural and animal rearing practices. Precautions are highly recommended to avoid any public health hazards in future.

KEY WORDS: ALBAHA, CHEMICAL, COLIFORM BACTERIA, SPATIAL, PHYSICAL, PARAMETERS, WATER QUALITY .

INTRODUCTION

Water is considered to be an important element to the life on the earth (Szewzyk et al., 2000). Although, the importance of freshwater for the consumption and other different uses, many diseases can be transmitted by this important type of water (Hahn, 2006; Ozler & Aydin, 2008). In addition, freshwater represents the only 3% of the total water resources in the world, which is found in groundwater, lakes, and rivers (APHA, 2012). In rural area, groundwater is preferred for the purpose of drinking due to its quality and safety in comparison to surface water. However, increased human activates affected this groundwater quality and added lots of chemical, physical, and microbial pollutants(Okeola et al., 2010; Patil et al., 2012). In addition, the quality of groundwater can be influenced by other different factors, such as geology, weathering system, the amount of recharge water and the

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interaction between rock and water (Sethy et al., 2016, Behailu et al 2018, Bourke et al., (2019).

In the ground aquifers, hand-dug wells (ancient wells) can be created. These types of wells varied in both depth and the volume of water, and can be exposed to various chemical, physical and bacterial pollutants (Yakubu, 2013). One of the most important keys to assess the quality of groundwater is to measure its chemical composition. Increased concentrations of chemical elements in drinking water can result in critical health hazards, (Mora et al., 2017). For example, methemoglobinemia can be caused by consuming water containing high concentrations of nitrate (Fan & Steinberg, 1996). Also, a laxative gastrointestinal disturbance could be triggered by consuming water containing high levels of sulfate(WHO, 2017), while the risk of hypertension can be increased through the consumption of ground water with high concentrations of salts(Chao et al., 2016).Generally, water-related diseases influenced millions of people every year especially children (Kisaka & Mato, 2018).

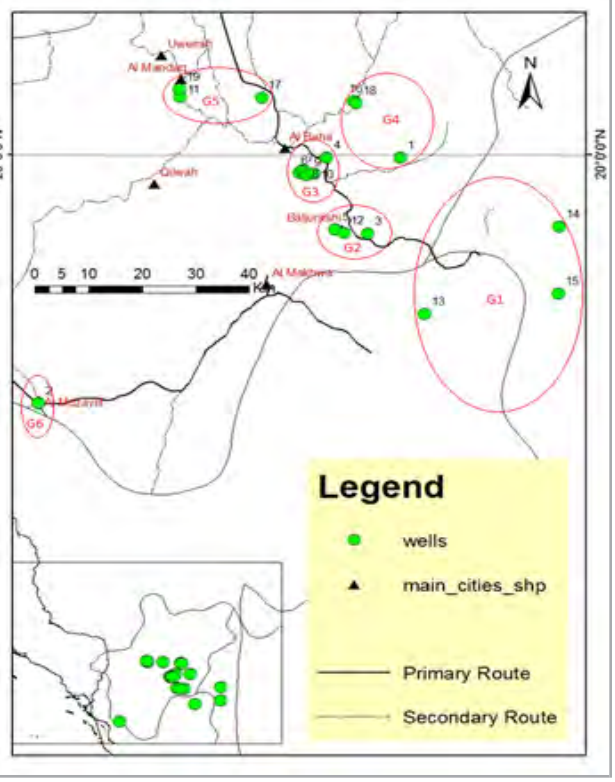
On the other hand, bacterial contamination of wateris the underlying cause of numerous water-borne diseases. Pathogenic bacteria should not be presented in drinking water. So, bacterial evaluation of groundwater intended to be consumed by human is an important health precaution(WHO, 1993). Coliform bacteria, including Escherichia coli (*E. coli*), have long been used as an indicators of water contamination. Coliform bacteria consist of total coliform bacteria and fecal coliform bacteria. Fecal coliform bacteria, such as *E.coli* are founded in the intestine of human and animals and their presence in water may indicate deposition of fecal materials. Consequently, drinking water should be tested for the presence or absence of coliform bacteria especially *E. coli* to guarantee its safety for human consumption (WHO, 2008).

The aims of this research are to (i) measure the concentrations of some physical, chemical parameters and also to screen for coliform bacteria and *E. coli*in some wells in Albaha region and surrounding area, (ii) compare these parameters with Saudi standards and WHO (World Health Organization) guidelines for drinking water quality, (iii) determine the effect of spatial variation on the concentrations of environmental parameters, and (iv) determine the relationships between environmental parameters.

MATERIAL AND METHODS

The study sites: Nineteen wells were randomly selected from different zones in Albaha region and its surrounding area(Saudi Arabia) during 2017, (Figure 1) and (Table 1). Well sites were divided into 6 groups based on geographical locations (Figure 1) and (Table 2). Albaha region consists of different villages and almost half a million of population. In this region of Saudi Arabia, groundwater wells have long been used as the main source for drinking water and irrigation(Omer et al., 2014).

Figure 1: Geographical location of targeted wells (n=19) in six sites (as groups of wells, G1-G6) in Albaha region and its surrounding area (Saudi Arabia).



Sample collection: According to previously published methods(APHA, 1998; Behailu et al., 2018), water samples were collected from 19 wells during 2017 using sterile 100 mL bottles for bacterial examination,and polyethylene bottles for physical and chemical tests. Each sample was labeled and transported, and stored at 4 ^{0}C until analysis in the laboratory of Biological departments in the faculty of science and arts in Baljurashi. Environmental parameters were analyzed in the laboratory of public administration of water services in Albaha region.

Physical, chemical, and bacterial analyses: Physical parameters (Total dissolved solids and turbidity) were measured using conductivity meter and turbid meter (HQ14D, Hach, USA). Chemical parameters (Fe, Mn, SO_4, NO_3 and NO_2) were analyzed by spectrophotometer DR 2800 (Hach, USA), and pH meter PHS-25 (BANTE, China) was used to measure pH. EC blue 100p (HyServe, Germany)screening medium was used to examine the presence and absence of coliform bacteria.100 mL of the sample was put into coliform water test sampling container followed by addition of EC blue 100p and shaking for 10 second beforeincubation at 37 ^{0}C for 48 hours. Color changes of the mixture to green or blue color indicates the presence of coliform bacteria, while

no color change is indicative of coliform absence in the sample (JWWA, 2001; Kodaka et al., 2008).

Statistical analysis: The statistical package of SPSS, version 20 (IBM) was used to analyze all physical, chemical and bacterial data. Kolmogorov-Smirnov tests was applied and some data that were not normally distributed were square root transformed. One-way ANOVA was applied to test the significant differences in all environmental parameters between individual sites and sites as groups. Spearman's rank correlations (rs) was used to examine the relationships between environmental parameters (Bolter et al., 2002; Field, 2009).

Table 1. Numbers, names and locations of targeted wells in Albaha region and its surrounding area (Saudi Arabia).

Well no.	Names of wells (location)	Coordination		Altitude (m)
		Latitude	Longitude	
1	Bani Kabir (South of Albaha)	19.994561	41.660098	1906
2	Almudailif (West of Albaha)	19.534829	41.050467	47
3	Alshatebah (Baljurashi)	19.851837	41.604840	2026
4	Alqohqoh(Marasiaah)	19.995739	41.535219	2133
5	Albahri, (Baljurashi)	19.859957	41.549216	2037
6	Adaros (Bani Dabian)	19.966935	41.490027	2313
7	Sobaiq (Bani Dabian)	19.971517	41.495268	2275
8	Asfal Alwadi (Bani Dabian)	19.970397	41.499260	2304
9	Sadd Alwadi (Bani Dabian)	19.963037	41.503160	2410
10	Alhanashah (Bani Dabian)	19.965565	41.514047	2393
11	Sadd Manshiah (Almandag)	20.108782	41.288857	2189
12	Alhoson (Baljurashi)	19.854214	41.564965	2027
13	Hawallah (South east of Albaha)	19.702417	41.700848	612
14	Shawas (South east of Albaha)	19.865282	41.927247	1624
15	Sadd Nabah (Albashayer)	19.739621	41.926028	1785
16	Alzayetonah Shoop Alhalah (Albaha)	20.101866	41.580797	1857
17	Alfaraah (Almosa)	20.107243	41.426129	2224
18	Alzayetonah, Shoop Alhallah (Albaha)	20.097328	41.585645	1866
19	Almaared (Almandag)	20.123787	41.288205	2151

Table 2. Sites as groups in Albaha region and its surrounding area, Saudi Arabia

Group number	wells no.	Group name	Symbol
1	13, 14 and 15	South east of Albaha	SA
2	3, 5 and 12	Baljurashi	BA
3	4, 6, 7, 8, 9 and 10	Bani Dabian	BD
4	1, 16 and 18	East of Albaha	EA
5	11, 17 and 19	Mandag	MA
6	2	Mudailif	MO

RESULTS AND DISCUSSION

Water used for different purposes, such as drinking and irrigating should be tested for its quality. According to these purposes, one of the important key role to assess the quality of water is to select the required parameters to be tested (Al-Hasawi et al., 2018). In this study, all parameters selection was based on their importance for determining quality of drinking water.

Comparison with Saudi standards and WHO guidelines of drinking water quality: Two physical (Total dissolved solids and turbidity) parameters, six chemical parameters (pH, Fe, Mn, SO_4, NO_3, NO_2), and also bacterial parameters are presented in Table 3 and Figures 2 to 18, respectively. All dissolved solids in water (manually mineral salts) are described as total dissolved solids (TDS) which are closely connected with conductivity (Iyasele et al., 2015). TDS ranged from 0.02 to 741 mg l^{-1} in sites 1 and 18, respectively with mean and SD values of 345.04 ± 218.58mg l^{-1}(Table 3 and Figure 2A & B). Approximately, 26.3% of wells (5 out of 19) exceeded the maximum concentrations of TDS recommended by Saudi standards (max. 500 mg l^{-1}) but all TDS values did not exceed the maximum concentrations recommended by WHO guidelines (max. 1000mg l-1) for drinking water quality. The slightly increases of TDS in sites 3, 3, 5, 16 and 18 may be as a results of both dissolving rocks in wells and application of fertilizers. Similar results have been documented by Rezaei & Hassani (2018) who assessed the quality of groundwater in the north of Isfahan, Iran and found that 14% of samples exceeded the maximum concentrations of TDS given by WHO.

The presence of turbidity in water with high values can affect other chemical and microbial parameters (WHO, 2004).Values of turbidity ranged from 0.14 to 3.51mg l-1 in sites 18 and 19, respectively (0.26 ± 0.43 mg l^{-1}) (Table 3 and Figure 2C&D). Only site 19 (5.3 % of all wells) exceeded the maximum concentrations of turbidity recommended by Saudi standards for drinking water quality (max. 1 NTU), while all concentrations of turbidity were below the maximum concentrations recommended by WHO guidelines (max. 5 NTU). This turbidity may be attributed to the presence of clay, silts, suspended solids, plankton and other microbes. Armah (2014) found turbidity is one of the most significant factors predict total coliform bacteria in mining environment in Ghana.

The pH values ranged from 7.34 to 8.67 SU in sites 14 and 11 (7.98 ± 0.37 SU) (Table 3 and figure 2E &tF). Sites 11 and 8 (10.5% of the total wells) exceeded slightly the maximum concentrations of pH recommended by Saudi standards and WHO guidelines (max. 8.5 SU) for drinking water quality, while the other values of the other sites were within the recommended range (6.5 – 8.5 SU). The result of all values of pH in this study including sites 11 and 19 reflect that groundwater was slightly basic which is the same result obtained by Al-Hasawi et al.(2018) in the study of groundwater in Rabigh governorate, Saudi Arabia.

Fe is one of the most abundant element on the earth crust. The more ion in water the more inconvenient for human consumption since it produces undesirable color and taste(Zogo et al., 2010). Values of ferrous (Fe) ranged from 0.01 to 0.3 mg l^{-1} in sites 18 and 5 (0.13 ±0.10mg l^{-1}) (Table 3 and figure 3A &B). No values of Fe exceeded the maximum concentrations recommended by Saudi standards and WHO guidelines for drinking water quality (max. 0.3 mg l^{-1}). This result is in consistent with the results obtained by Kisaka & Mato (2018) who found the Fe within the permissible concentration in groundwater in Tanzania.

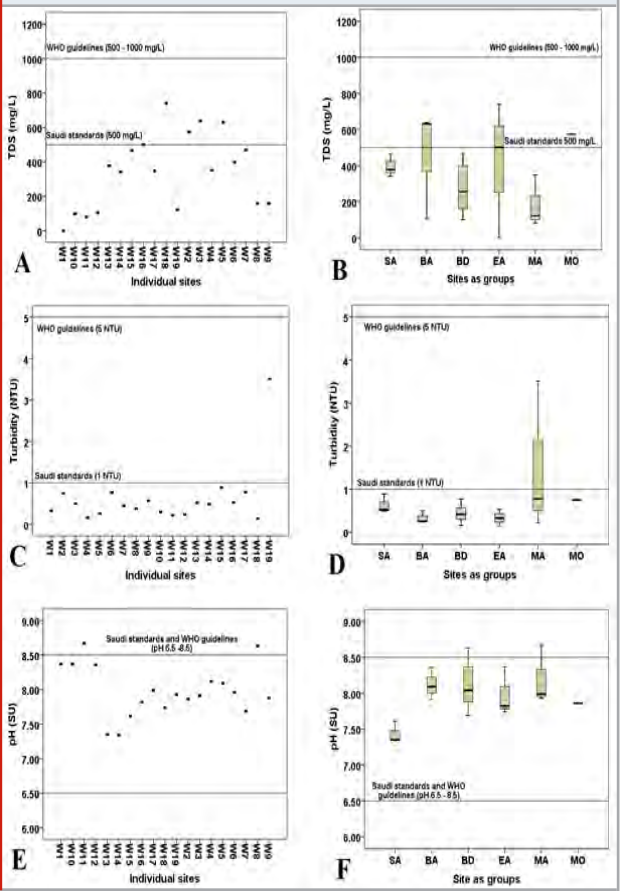
Table 3. Details of some physical, chemical and bacterial parameters of targeted wells in Albaha region and its surrounding area.

Well no.	Parameters								Coliform bacteria
	pH	TDS (mg l^{-1})	Turbidity (NTU)	Fe (mg l^{-1})	Mn (mg l^{-1})	SO_4 (mg l^{-1})	NO_3 (mg l^{-1})	NO_2 (mg l^{-1})	
1	8.37	0.02	0.330	0.03	103	1.12	240	0.020	–
2	7.86	573	0.750	0.02	0.6	81	1.1	0.040	+
3	7.91	638	0.500	0.23	0.1	75	3.1	0.020	+
4	8.12	351	0.160	0.15	0.3	64	0.5	0.001	+
5	8.09	629	0.260	0.26	0.01	629	2.7	0.020	–
6	7.96	399	0.770	0.14	0.1	399	1.5	0.012	–
7	7.69	469	0.450	0.03	0.7	469	0.2	0.018	–
8	8.63	159.4	0.380	0.22	0	123	0.3	0.014	+
9	7.88	158.5	0.570	0.1	0.1	16	0.7	0.010	–
10	8.37	99.7	0.300	0.02	0.3	9	3.3	0.020	–
11	8.67	80.7	0.220	0.23	0.2	21	23.9	0.004	+
12	8.36	104	0.240	0.16	1	7	3.3	0.010	–
13	7.35	377	0.520	0.05	0.2	32	2.5	0.044	+
14	7.34	341	0.490	0.3	0.1	34	0.4	0.022	–
15	7.61	466	0.890	0.1	0.1	75	1.3	0.010	–
16	7.82	501	0.530	0.3	0.5	38	1.9	0.005	+
17	7.99	347	0.780	0.02	0.7	59	3.8	0.002	–
18	7.74	741	0.140	0.01	0.1	113	1.7	0.017	–
19	7.93	121.4	3.510	0.05	0.9	33	709.8	0.009	–
Mean ± SD	7.98 ± 0.37	345.04 ± 218.58	0.62 ± 0.43	0.13 ± 0.10	5.74 ± 23.56	119.90 ± 176.46	52.74 ± 168.20	0.157 ± 0.011	NA
Range	7.34 – 8.67	0.02 - 741	0.14 - 3.51	0.01 - 0.3	0 - 103	1.12 - 629	0.2 - 709.8	0.001 - 0.044	NA
KSA PL	6.5 - 8.5	500	1	0.3	0.4	250	50	0.2	–
WHO PL	6.5 - 8.5	500 - 1000	5	0.3	0.1 - 0.5	250	50	3	–

WHO: world health organization, PL: permissible limits, SD: standard deviation, NA: not applicable.

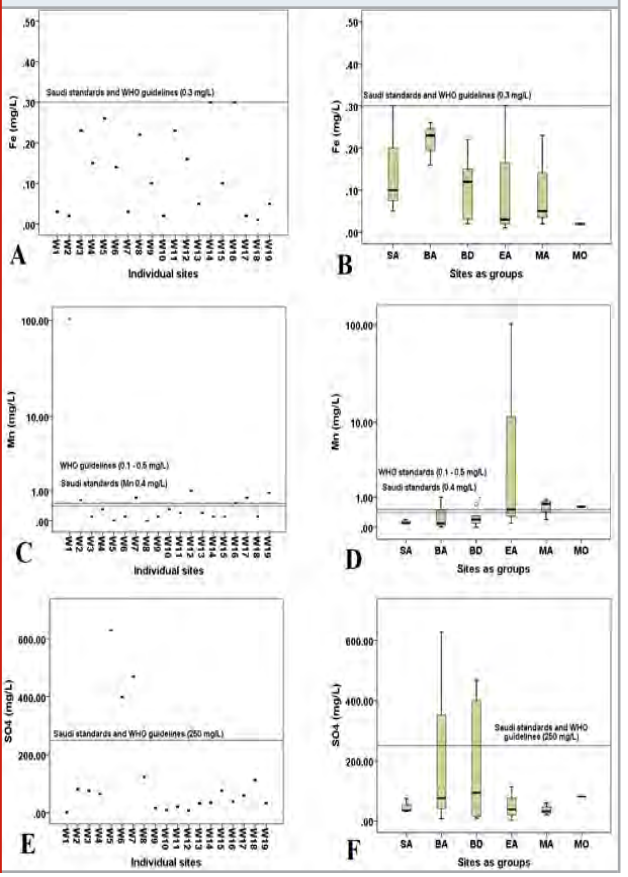
Manganese (Mn) can be found naturally in groundwater resulting from soluble of bedrock and also it can be leached into groundwater from human practices (Ljung & Vahter, 2007). Mn values ranged from 0 to 10_3 mg l^{-1} in sites 8 and 1 (5.74 ± 23.56 mg l-1) (Table 3 and figure 3 C & D). Approximately, 31.6% of the total wells (sites; 1, 2, 7, 16, 17 and 19) slightly exceeded the maximum concentrations of Mn recommended by Saudi standards for drinking water quality. Similarly, all sites mentioned above except site 16 (26.3% of total wells) slightly exceeded the maximum concentrations of WHO guidelines. The large value of Mn was recorded in site 1(103 mg l^{-1}) in Bani Kabir (Table 1& Figure 1).Increased Mn levels in groundwater (above 0.4 mg l^{-1}) can be risky to health (WHO, 2017). High levels have been documented in studies elsewhere and attributed to both intensive agriculture practices and Mn ions naturally found in groundwater (Phan et al., 2013; Van et al., 2016).

Figure 2: Variation of TDS (A & B), turbidity (C & D), and pH (E & F) between individual sites and sites as groups in some wells in Albaha region and its surrounding area, Saudi Arabia during 2017.



Sulfate (SO_4) can be found in groundwater from both natural and anthropogenic sources (in the form of fertilizers). Water–related diseases, such as diarrhea, especially to young children, can be caused by high concentrations of SO_4 in drinking water (Kisaka & Mato, 2018; Miao et al., 2012). Values of sulfate (SO_4) ranged from 1.12 to 629 mg l^{-1} in sites 1 and 5 (119.90 ± 176.46mg l-1) (Table 3 and figure 3E& F). Three out of 19 wells (15.8%) (sites; 5, 6 and 7) exceeded the maximum concentrations of SO4 recommended by Saudi standards and WHO guidelines for drinking water quality. The study of the source of SO4 in groundwater in the Jinghuiqu district (China) found increases of SO4 during the period of time from 1990 to 2009 mainly due to dissolution of minerals(Liu et al.,2012).

Figure 3: Variation of Fe (A & B), Mn (C & D), and SO_4 (E & F) (mg l^{-1}) between individual sites and sites as groups in some wells in Albaha region and its surrounding area, Saudi Arabia during 2017.



Nitrate (NO_3) can easily reaches the groundwater through agricultural activities, sewage contamination, and from the atmosphere (Ritzi et al., 1993). Nitrate (NO3) values ranged from 0.2 to 709.8 mg l^{-1} in sites 7 and 19 (52.74 ± 168.20mg l-1) (Table 3 and figure 4 A & B). Two sites (10.5% of total wells) showed elevated values of NO3exceedingthe maximum concentrations recommended by Saudi standards and WHO guidelines for drinking water quality. The highest values were in site 19 (709.8 mg l^{-1}) followed by site 1 (240 mg l^{-1}). The increased concentrations of NO3 in sites 1 and 19 may had resulted from agriculture practices through applying animal manure and inorganic nitrogenous fertilizer discharging into water wells. Recent study of the source of NO3 in groundwater conduct by Bourke and associates (2019) concluded that increased NO3 most likely attributed to mixing or denitrification and also agriculture activities.

Figure 4. Variation of NO_3 (A & B) and NO_2 (C & D) (mg l^{-1}) between individual sites and sites as groups in some wells in Albaha region and its surrounding area, Saudi Arabia during 2017.

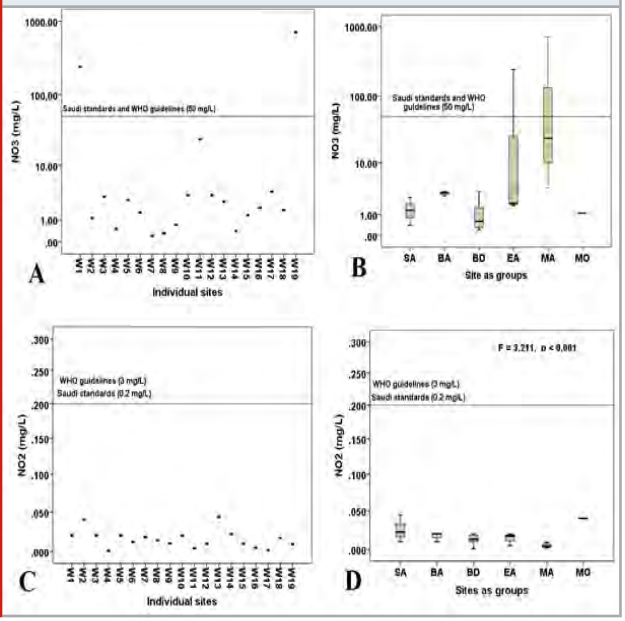
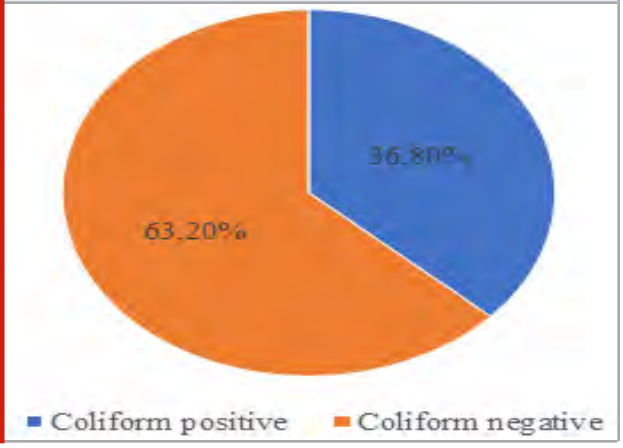


Figure 5: Percentage of coliform presencein individual sites in some wells in Albaha region and its surrounding area, Saudi Arabia during 2017.



Nitrite (NO_2) values ranged from 0.001 to 0.044 mg l^{-1} in sites 4 and 13 (0.157 ± 0.011mg l-1) (Table 3 and figure4 C&D). No wells exceeded the maximum concentrations of NO2 values recommended by Saudi standards and WHO guidelines for drinking water quality. The NO_2 can be naturally found in groundwater as a result of nitrogen cycle (Nas & Berktay, 2005).

Table 4. Relationship between environmental parameters in some wells in Albaha region and its surrounding area

Parameter	pH	TDS (mg l^{-1})	Turbidity (NTU)	Fe (mg l^{-1})	Mn (mg l^{-1})	SO_4 (mg l^{-1})	NO_3 (mg l^{-1})	NO2 (mg l^{-1})
pH	–	rs = -.574*, p < 0.05	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
TDS (mg l^{-1})	rs = -.574*, p < 0.05	–	N.S.	N.S.	N.S.	rs = .771**, p < 0.001	N.S.	N.S.
Turbidity (NTU)	N.S.	N.S.	–	N.S.	N.S.	N.S.	N.S.	N.S.
Fe (mg l^{-1})	N.S.	N.S.	N.S.	–	N.S.	N.S.	N.S.	N.S.
Mn (mg l^{-1})	N.S.	N.S.	N.S.	N.S.	–	rs = -.500*, p < 0.05 p < 0.05	rs = .457*,	N.S.
SO_4 (mg l^{-1})	N.S.	rs = .771**,	N.S.	N.S.	rs = -.500*,		rs = -.535*,	
		p < 0.001			p < 0.05	–	p <0.05	N.S.
NO_3 (mg l^{-1})	N.S.	N.S.	N.S.	N.S.	rs = .457*, p < 0.05	rs = -.535*, p <0.05	–	N.S.
NO_2 (mg l^{-1})	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	–

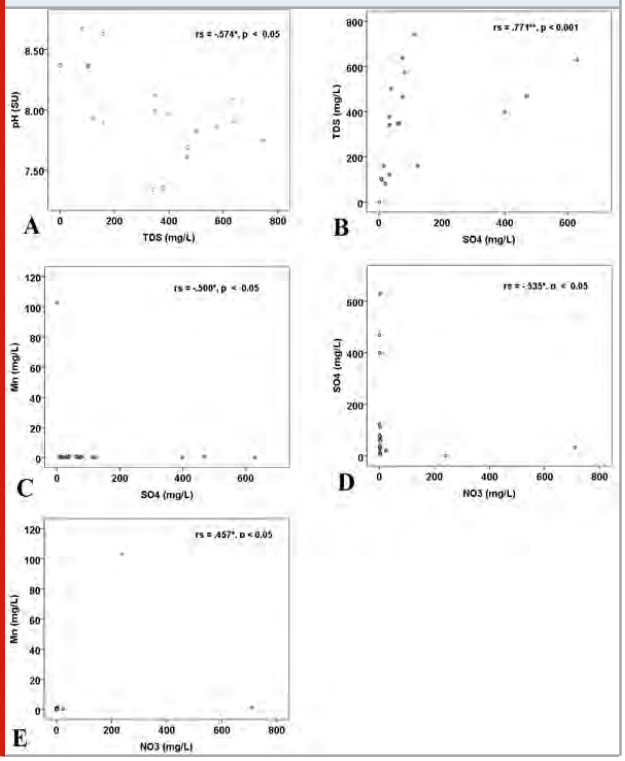
Key symbols: SU: standard unit, N.S.: not significant, rs: Superman's rank correlation.

Results of EC blue analysis are presented in Table 3and Figure 5. Approximately, 36.8% of wells (sites; 2, 3, 4, 8, 11, 13 and 16) are contaminated with coliform bacteria. These pathogenic bacteria should not be founded in water used for drinking purpose and their viable count should be 0 per 100 mL (RCER, 2015; SASO, 2000; WHO, 2004, 2017).

The presence of coliform bacteria and *E. coli* in the wells mentioned above may resulted from deposition of animal manure, sheep and goats faces around the opened wells especially after rainfall events. Sakami et al.(2003) stated that groundwater receives pathogens from animal manure after rainfall events. A recent study revealed that all water sources including wells used for drinking purpose except tanks, in Baljurashi city, Albaha region

were contaminated with coliform bacteria and E coli(Omer et al., 2014). It should be noted that positive results of coliform bacteria assay do not necessarily reflect a fecal contamination of water. All coliform genera, except *E. coli*, have been isolated from natural samples of non-fecal origin and all genera show a positive reaction in this assay (Doyle & Erickson, 2006).

Figure 6: Relationship between pH (SU) – TDS (A), TDS– SO_4 (B), Mn– SO_4 (C), SO_4– NO_3 (D) and Mn– NO_3 (E) in some wells in Albaha region and its surrounding area, Saudi Arabia during 2017.



Effects of spatial factors on the variation of environmental parameters: It is important to study of spatial effects on variations of physical, chemical, and microbial parameters. These parameters can be driven by geology, climate conditions and human activities (Ali & Ali, 2018). Effects of spatial factors on the variation of physical, chemical and bacterial parameters are presented in Table 2 and figures 1 & 17. One–Way ANOVA analysis shows no significant effects of individual sites on the variation of all environmental parameters investigated. However, sites as groups revealed significant effect on the variation of only nitrite (NO_2) ($F=3.21$, $p< 0.001$) in group 1 (SA) (see figures 1 & 17 and Table 2). Additionally, all values of NO_2 at all sites do not exceed the maximum concentrations recommended by WHO guideline and Saudi standards for drinking water quality. The slight increases ofNO2 concentrations in group 1(SA) compared with other groups in this research may be due tonaturaleffect as a part of nitrogen cycle(Bourke et al., 2019; Nas & Berktay, 2005).

Significant relationships between environmental parameters: Previous studies of groundwater contamination had emphasized on the interplay between different environmental parameters by employing correlation coefficient statistics. This type of statistical analysis helps researchers to find out how environmental parameters effect water quality and accordingly how to manage strategies of water(Otu et al., 2014). Significant sportsman's correlations coefficient analysis between all environmental parameters are presented in table 4 and figures 19 to 23.pH was negatively correlated with TDS ($rs = - 0.574$, $p < 0.05$) (Figure 19). This result reflects that the more basic water the less TDS concentration and vice versa. This result differs with the result obtained by Mahato et al.(2018) who found a low positive correlation between pH and TDS in groundwater in eastern Terai region of Nepal. Results have also showed various significant correlations between SO_4 and different environmental parameters.

These results suggest that TDS, Mn and NO_3 influenced the SO_4. The SO was strongly positively correlated with TDS ($rs = 0.771$, $p < 0.001$) (Figure 20) and this observation is in good agreement with results of Salem & Alshergawi (2013) who studied51 wells used for drinking water in Alshati district of Libya. Also, SO_4 was negatively correlated NO3 ($rs = - 0.535$, $p < 0.05$) (Figure 23). Similar result obtained by Konget al. (2013) but in different environment (atmospheric particles). In contrast, Kim et al.(2005) observed a positive correlation between SO_4 and NO_3 in groundwater in Namwon, Korea. The SO_4 was also negatively correlated with Mn ($rs = - 0.500$, $p<0.05$) (Figure 21). Similar weak negative correlation in groundwater was found by Shroff et al.(2015) in Valsad district of south Gujarat, India. Moreover, Mn was positively correlated with NO_3 ($rs = 0.457$, $p <0.05$) (Figure 22). Other studies, for example, Bishnol & Malik (2008) and Salem & Alshergawi (2013) found no relationships between Mn and all the investigated environmental parameters including NO_3 in groundwater.

Overall, physical chemical and bacterial parameters were studied in Albaha region and its surrounding area, Saudi Arabia. More than two thirds of wells are unsuitable for drinking and irrigation purposes due to inconsistencies with Saudi and WHO standards in terms of physical, chemical, and bacteriological parameters. Increases in TDS, turbidity, pH, SO_4, NO_3 and Mn, were found along with coliform bacteria. No statistically significant variations of environmental parameters were observed between individual well sites. Significant correlations were found between TDS and sulfate levels, Mn and nitrate, pH and TDS, and sulfate and nitrate. Urgent treatment for wells in Albaha region is strongly advised. Further investigations of groundwater quality in terms of temperature, heavy metals contents are highly recommended.

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Phytochemical Profile of *Phyllanthus niruri* L and evaluation of its Potent Bioactive Compounds

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ABSTRACT

*Phyllanthus niruri*L. is one of the highly used medicinal plants both traditionally and scientifically. Phytochemical screening of such plants is of high importance to establish the claims of medicinal uses by traditional and folk medicine practitioners. The phytochemical screening reveals the presence of potent bioactive compounds that can be effectively used for the preparation of better herbal drugs. The study aims at determining the potent bioactive compounds present in the leaf extract of *Phyllanthus niruri* L. utilizing qualitative analysis and HPLC analysis with standard as well as evaluate the antimicrobial potential of such extract. This done means of Soxhlet extraction of dried plant leaves. The extracts are screened by standard protocol to determine bioactive compounds. Further selective and confirmed detection of bioactive compounds is done employing HPLC using standard compounds of phenol, flavonoid, lignans, and alkaloid. The extracts were also evaluated for their antimicrobial potential against bacteria (Staphylococcus aureus and Bacillus subtilis) and fungi (Aspergillus niger and Candida albicans). The overall result obtained from the study showed the presence of potent bioactive compounds in extracts of *Phyllanthus niruri* L., such as quercetine, rutin, phyllanthin, and ellagic acid. The extract also showed the effective antimicrobial potential against both bacteria and fungi. The study derives the conclusion that the plant *Phyllanthus niruri* L. is a rich source of potent bioactive compounds and hence carries out several important biological activities. The presence of such essential phytochemicals in its extract is the reason behind its historical and traditional use in medicines..

KEY WORDS: PHYLLANTHUS NIRURI PHYTOCHEMICAL SCREENING, HPLC, ANTIMICROBIAL ACTIVITY.

INTRODUCTION

Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world (Igbinosa et al., 2009). In the Democratic Republic of Congo (DRC), among the species used in the treatment against malaria, *Phyllanthus niruri* L.is well-positioned for different previous studies on this plant (Pauwels, 1993; Tona et al., 1999 and Cimanga et al., 2004). Medicinal plants are now getting more attention than ever because they have the potential of myriad benefits to society or indeed to all mankind, especially in the lien of medicine and pharmacological studies. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun et al., 2007 Gupta and Vaghela (2019 Meselhy et al 2020).

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Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds and many more (Edeoga et al., 2005). Due to their specialized biochemical capabilities, plants can synthesize and accumulate a vast array of primary and secondary chemicals useful for the plant itself as protecting against environmental stress factors. These compounds have made many plants useful also for humans, for instance, spices and medicines, etc. (Akinmoladun et al., 2007 and Edeoga et al., 2005).*P. niruri* L. is one of the most important medicinal plant used in different regions in the world for the treatment of various diseases such as jaundice, asthma, hepatitis, flu, dropsy, diabetes, fever causing by malaria (Kerharo and Adam, 1974; Ishimari et al., 1999; and Paran-jape, 2001) but its availability is drastically decreasing because of numerous harvests.

Phyllanthus is a large genus of shrubs, trees and rare herbs of the family Euphorbiaceae, comprising more than 600 species. The genus is found in almost overall warmer parts of the world (Burkil, 1966). Among the Phyllanthus species, *P. niruri* is a small erect annual herb growing up to 30–40 cm in height and is indigenous to the Amazon rainforest and other tropical areas, including South East Asia, Southern India and China (Girach et al., 1994).*P. niruri* L.has been the subject of much research to investigate the active constituents and their pharmacological activity, beginning in the mid-1960s. Row et al., 1964 was the first to work on *P. niruri* L. and reported the isolation of phyllanthin. It has a rich source of phytochemicals, many of which have been found only in *P. niruri* L.(Dhar et al., 1968 Nisar et al 2018, Mehta et al 2019, Meselhy et al 2020).

The aerial part of *Phyllanthus niruri* L. has been used by many countries in folk medicine for the treatment of various disease conditions such as increase libido or fertility in men. In India, the plant is usually used by traditional medicine practitioners for the treatment of asthma, bronchial infection, liver diseases, diabetes, gonorrhea, inducing labor and treatment of edema, feverish pain, sore throat, female sterility, oliguria, and vaginitis. They also used the plant to manage irregular menstruation, tachycardia, dysentery, spasmodic cough, itchiness, arthritis, otitis, swelling, skin ulcer and weakness of male organ (Obianime and Uche, 2009). In Brazil, the tea of *Phyllanthus niruri* L. is used to treat renal calculi (Nishiura et al., 2004). In South Africa, it is used in folk medicine to treat hyperuricemia (Murugaiya et al., 2009 Gupta and Vaghela (2019).

The extract of *Phyllanthus niruri*L. has shown several pharmacological activities. Ethanolic extract of *Phyllanthus niruri* L. was found to have significant antidiabetic activity in insulin-dependent diabetes mellitus rats but showed no effect on non-insulin-dependent diabetes mellitus rat (Bavarva and Narasimhacharya, 2007). *Phyllanthus niruri*L.has shown an inhibitory effect against calcium oxalate crystal growth and aggregation in human urine. This medicinal plant exhibited antiurolithic activity in both in vitro and in vivo studies (Barros et al., 2003). Scientific studies have shown that *Phyllanthus niruri* L.has an antihyperlipidemic effect. It was also reported that the aqueous extract exhibited antihyperlipidemic activity (Nwanjo et al., 2007 Mehta et al 2029, Meselhy et al 2020).

Meselhy et al. (2020) highlighted the direct methods used for extraction of lignans from aerial parts of *P. niruri* L. Identified lignans were phyllanthi, hypophyllanthin, phylltetralin, nirtetralin, and niranthin. Different solvents gave yield based on extraction concentrations. At 18.10 g % (w/w), aqueous extract yielded phyllanthin of 0.33 ± 0.10 mg/g extract), while at 3.6 g% w/w, methanolic extract yielded comparatively lower phyllanthin content of 3.1 mg/g extract. Soxhlet method of extraction also yielded at concentrations of 0.82, 1.12, and 3.40 g% w/w of hexane, dicholoromethane or acetone yielded higher phyllanthin contents of 36.2 ± 2.6, 11.7 ± 1.68, and 11.7 ± 1.10 mg/g. Alkaline digestion was employed to obtain high phyllanthin content 22.34 ± 0.13 mg/g at 3.1 g% w/w, Microwave-assisted extraction method yielded 21.2 ± 1.30 mg/g content and plant material treatment at 50˚C with two hydrolytic enzymes, cellulase (9 U/g for 12 h) and protease (4 U/g up to 72 h) yielded phyllanthin content 25.9 mg/g extract. Ethanolic extract of *Phyllanthus niruri* L. was found to have potential antiplasmodial activity in vitro by inhibition of the developmental stage of a trophozoite to schizonts. Another study showed that *P. niruri* exhibited potent systemic antinociceptive actions against two models of neurogenic pain (Santos et al., 1995 Meselhy et al 2020).

In recent years, excessive use of drugs has made most of them resistant against popular antibiotics. Plants have a rich source of active components and have upper hand over chemical compounds owing to their severe side effects. In consideration of the present scenario, this study highlights the active components analysis of *Phyllanthus niruri* l. It would help to identify its antibacterial and antifungal potency and thus used to curb their growth. The aim of the current study was to carry out the phytochemical profiling of *Phyllanthus niruri* L. through Liquid chromatography and to obtain potent bioactive components from the work.

MATERIAL AND METHODS

Sample Preparation: *Phyllanthus niruri* L. leaves were collected from the Gopalganj district in Bihar. The leaves were washed with water to remove dust particles. The washed leaves were dried at room temperature and then powdered mechanically. The powdered leaves were extracted with the help of Soxhlet apparatus with methanol and petroleum ether as a solvent at 75°C for 6 hours. The extracts were dried and refrigerated at 4°C for further usage.

Phytochemical Screening: The phytochemical screenings were performed by following the standard procedures mentioned in Harbone (Harbone, 1998). Screenings for the presence of saponin, alkaloid, tannin, glycoside,

flavonoid, phenol, terpenoid and carbohydrates were performed for both the extracts.

Antimicrobial Assay: The antimicrobial activity of both the extracts of *Phyllanthus niruri*L. was assessed by the Kirby Bauer Disc Diffusion Method. The microbial strains used include Bacillus subtilis, *Staphylococcus aureus, Aspergillus niger, and Candida albicans*. For the test, Muller Hinton Agar Media was prepared and poured in Petri-dishes. After the solidification of agar media, 0.1 ml of each microbial strain was spread over the media. The discs were prepared with Whatman filter paper.Then the discs were saturated with the plant extract and left for drying. For sample preparation, 100µg/ml extract of *P. niruri* L. in the respective solvent was deposited onto the disc. After the disc was dry completely they were placed on the inoculated medium. The plates were incubated for 24 hours at 37°C for bacteria and 27°C for fungi. Levofloxacin for bacteria and Fluconazole for fungus were used as controls. After incubation, the zone of inhibition around the wells was detected, and the diameter of these inhibition zones was measured and recorded.

HPLC Analysis: The methanolic and petroleum ether extracts of *Phyllanthus niruri* L. were subjected to HPLC analysis of the instrument HPLC (Shimadzu VP 1605) with HiQSil C18-HS column (column size:4.6 mm × 250 mm × 5µM; 25°C), isocratic pump. The HPLC analysis of the extract was done to determine the presence of some specific phytochemical compounds in the extract. Acetonitrile was used as a solvent system for the elution of the sample. HPLC chromatograms were detected using a photodiode array UV detector at the scan range of 255nm-370nm phytochemical scan range according to the absorption maxima of analyzed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The standard phytochemical compounds used, the mobile phase used for them and the detection parameter used is summarized in table 1. The stock solution of concentration 10µg/ml was prepared by dissolving 10µg standard in 0.5ml HPLC-grade methanol followed by sonication for 10 minutes and the resulting volume was made up to 1ml with the methanol. The standard and sample solutions were filtered through a 0.22µm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.

Table 1. Showing the standard and the condition used for the standard phytochemical compounds during the HPLC analysis

S. No.	Standard	Mobile Phase	Mobile Phase ratio	Detector	Flow rate	Run Time
1	Quercetin	Acetonitrile: Methanol	75:25	355nm	1.5ml/min	15min.
2	Rutin	Methanol	-	270nm	1.5ml/min	15min.
3	Ellagic acid	Methanol: Acetone	50:50	210nm	1.5ml/min	15min.
4	Gallic acid	Acetone: Methanol	55:65	240nm	1.5ml/min	15min.
5	Phyllanthus	Methanol: Acetone: Acetonitrile	25:50:25	210nm	1.5ml/min	15min.

Table 2. Showing the result for the phytochemical analysis of the plant extract for both solvents, where (+) indicates the presence of the compound and (-) indicates the absence of compound

S. No.	Phytochemical compound	Methanolic extract	Petroleum ether extract
1	Alkaloid	+	+
2	Saponin	+	-
3	Tanin	+	+
4	Glycosides	-	+
5	Terpenoids	+	+
6	Carbohydrates	-	-
7	Flavanoids	+	+
8	Phenols	+	-

Figure 1: Antibacterial activity of extract of *Phyllanthus niruri* L. against *B. subtilis* and *S. aureus*

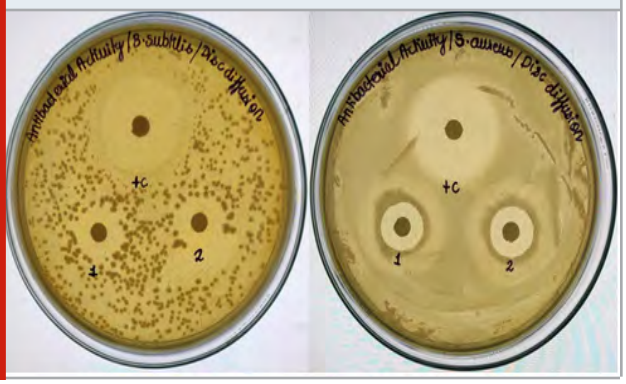
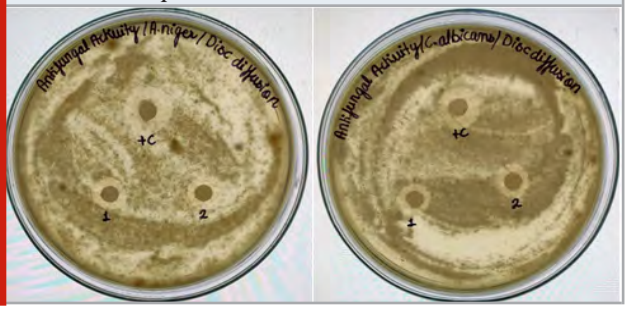


Figure 2: Antifungal activity of extract of *Phyllanthus niruri* L. against *A. niger* and *C. albicans* In the figure above disc 1 denotes methanolic extract and disc 2 denotes petroleum ether extract of *Phyllanthus niruri* L. and +C is for antibiotic positive control



RESULTS AND DISCUSSION

Herbal medicine has been used for centuries for the treatment of various diseases. It is an important part of Ayurveda, Siddha, and Unani medicine. Different parts of various plants are used by indigenous people across the world to cure wounds, snakebites, abdominal pain, skin infections, and several other diseases. In a study by WHO, it was estimated that 80% of the world population still depend on herbs and plants as medicine (WHO, 1991). Several phytochemicals such as vincristine, artemisinin, quinine, and digoxin have been isolated from plants which have shown a broad range of pharmacological activities (Eslami et al., 2017; Adedinsewo et al., 2017). This study aimed at phytochemical screening and antimicrobial activity of *Phyllanthusniruri* L. Phytochemical Screening: The phytochemical screening of methanolic and petroleum ether extract of the plant was carried out by standard procedure. The result obtained is summarized in table 2. It showed the presence of phenol, flavonoid, saponin, alkaloid, and terpenoid. These compounds are responsible for antioxidant activity and antibacterial activity.

Antimicrobial activity: The extracts of *Phyllanthus niruri* showed good antimicrobial activity against the selected microbial strains. The extract carried out potential antimicrobial activity due to the presence of bioactive compounds like flavonoids and phenols in them. The result of the antimicrobial activity of both extracts is summarized in table 3.

HPLC Analysis: The HPLC analysis of both the extracts revealed the presence of some potent phytochemical compounds in the plant extract. The phytochemical compound confirmation was done with the help of standard. The confirmation basis is the retention time at which the standard phytochemical compound gave the peak. The similarity in peak formation time between sample and standard is indicative of the presence of that phytochemical compound in sample extract. The result depicted the presence of flavonoids like quercetin and rutin, phenols like ellagic acid, alkaloid like securinine and lignan like phyllanthin; in both the extracts. The chromatograms and results of HPLC analysis of the sample and standard obtained are given below. The retention time peak shown by the entire standard compound is also found in the sample extract.

Table 3. Showing the result for the antimicrobial activity of the plant extract for both solvents against the respective microbes, the table depicts the Zone of Inhibition obtained against each extract

S. No.	Microbe	Zone of Inhibition (mm)		
		Methanolic extract	Petroleum ether extract	Positive control
1	*Bacillus subtilis*	17	16	26
2	*Staphylococcus aureus*	14	16	27
3	*Aspergillus niger*	11	13	18
4	*Candida albicans*	10	11	17

The modern sedentary lifestyle, stress, pollution, junk food, and alcohol have exacerbated the harms caused by free radicals. The free radicals are associated with diseases such as diabetes, arthritis, cancer, Parkinson's disease, and Alzheimer's disease (Kaur and Kumar, 2016; Meena et al., 2018). The presence of phytochemical compounds in plant extract of *Phyllanthus niruri* L. was determined by qualitative analysis as well as by HPLC. The analysis revealed the presence of phytochemicals like phenol, flavonoid, alkaloid, and lignans the extracts can scavenge free radicals. These compounds have previously shown strong anticancer, antidiabetic, anti-inflammatory and antimicrobial activity (Nyamai et al., 2016). The bioactive compounds present in the extract are full of potential medicinal properties and hence gain a lot of attention for use in the herbal medication process.

Phenols and flavonoids have significant antioxidant properties. Phenols are also associated with the ability to inhibit the growth of bacteria (Chan et al., 2011). Rutin is important because it strengthens capillaries and so can help people suffering from arteriosclerosis or high blood pressure (Becker et al., 1985) while quercetin has anti-aggregant, anticancer, anti-fungal (especially

anti-dermatophytes), antifeedant, anti-glaucomic, anti-inflammatory, anti-oxidant, antiseptic and anti-spasmodic activity (Saija et al., 2003).

Figure 3: HPLC chromatogram of *Phyllanthus niruri* L. [A] Methanolic Extract [B] Petroleum ether extract

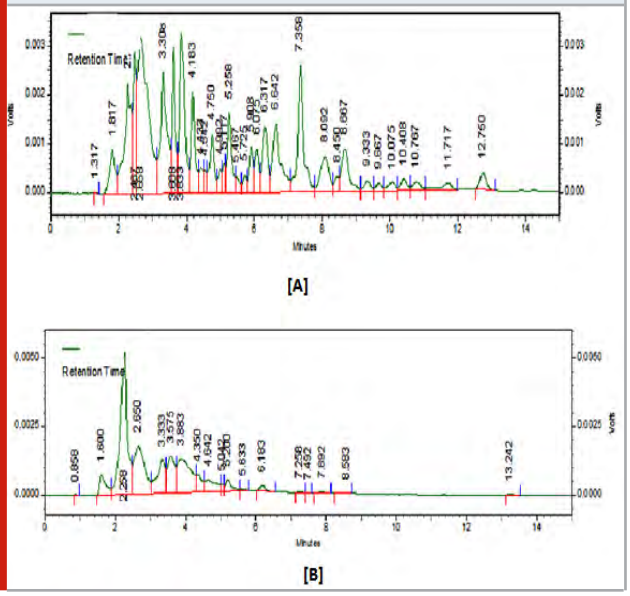
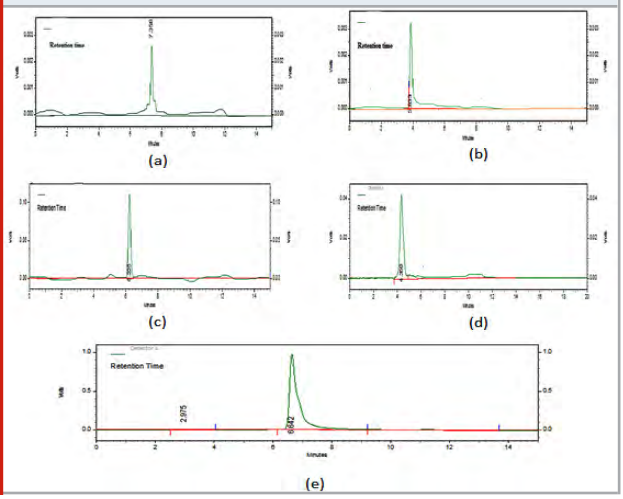


Figure 4: HPLC Chromatograms of standard compounds showing single peak (a) Quercetin chromatogram (Rt – 7.358 min) (b) Rutin chromatogram (Rt – 3.833 min) (c) Ellagic acid chromatogram (Rt – 6.225 min) (d) Securinine chromatogram (Rt – 4.360 min) (e) Phyllanthin chromatogram (Rt – 6.642 min)



Furthermore, these compounds have shown anti-inflammatory, anticancer and antidiabetic activity. The presence of these compounds forms the basis of antioxidant and antimicrobial properties of the *P. niruri*L. extract. Phenolic compounds may play an important role in preventing chronic illnesses such as cardiovascular disease, a certain type of cancers, neurodegenerative disease, and diabetes (Verma et al., 2010). Flavonoids have been shown to possess many pharmacological properties such as anti-oxidant activities, anti-inflammatory activities, anti-cancer activities, and anti-microbial effects, hence, flavonoids may have a contributory effect to its fertility properties and other pharmacological effects the plant possesses (Verma et al., 2011 and Harbone, 1998). The study was aimed to perform the phytochemical analysis and determine antioxidant potential of *P. niruri*. Mehta et al. (2019) in their study considered qualitative and quantitative characterization of phytochemicals and antioxidants present in *P. niruri* extract. It was observed from the results that aqueous extract of the plant possessed high antiodixant activity that could be by virtue of flavonoids present in it.

Table 4. Showing the summarized result of the HPLC chromatogram analysis of the standard phytochemical compounds

S.No.	Standard name	Retention Time	Area	Area %	Height	Height %
1	Quercetine	7.358	144977	100	21456	9.076
2	Rutin	3.833	244977	100	21456	9.076
3	Ellagic Acid	6.225	144977	100	21456	9.076
4	Securinine	4.360	2135442	97.133	203445	94%
5	Phyllanthin	6.642	942696	94	45062	943

Phyllanthin belongs to the lignanscategory and has been shown to possess hepatoprotectiveand anti-genotoxic activities (Row et al., 1964). Phyltetralin, nirtetralin, and niranthinextracted from *Phyllanthus niruri*L. exhibited anti-inflammatory activity by inhibiting carrageenan-induced paw edema and neutrophil influx (Kassuya et al., 2005).The HPLC analysis of the sample is revealing the presence of potent phytochemicals compounds in both plant extracts indicating that the plant extracts have good medicinal value as reported by Meselhy et al., (2020). The emergence of antibiotic-resistant bacteria is another major health concern globally. The search for novel antioxidant and antimicrobial compounds is carried out throughout the globe by scientists meticulously. Phenol, flavonoids, and tannins are a major class of phytochemicals that have antimicrobial activity (Cowan et al., 1999; Gupta and Vaghela, 2019). The plant extract of *Phyllanthus niruri* L. has shown the good

antimicrobial property as well hence their introduction in herbal medicine preparation might be an effective approach (Nisar et al., 2018).

CONCLUSION

The overall conclusion derived from the study is that *Phyllanthus niruri*L. is a source of potent bioactive compounds having essential and effective biological properties. *Phyllanthus niruri* L. is an important medicinal plant. The plant is widely used for the treatment of hepatic disease, edema, dropsical condition, and urinary troubles. *P. niruri*L.has many effective traditional uses for a wide variety of diseases. Some of the medicinal uses have been proven in experimental models, which suggest that the extracts of the plant possess various pharmacological actions. All the pharmacological properties shown by the extract of the plant is due to the presence of such bioactive compounds.

The study reveals the presence of such effective compounds. The study from the HPLC analysis has shown the presence of such bioactive compounds which carry out altogether a wide variety of biological functions. The study also concludes that these bioactive compounds impart good antimicrobial properties in the extracts as well. So the introduction of such an important plant in herbal drug formulation could lead to betterment in the effectiveness of drugs. Further studies could be carried out on various other solvent extracts of *P. niruri*L. as well as on determining another biological potential of extract than antimicrobial. Purification and incorporation of these bioactive compounds from *P. niruri*L. into medicinal use could a better idea for further study.

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Effects of Follicular Fluid Contamination by Microorganisms During *in vitro* Fertilization

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ABSTRACT

Follicular fluid (FF) is one of the important sources of micro-organisms that may affect IVF outcomes. IVF cultures were found to contaminate the fungi. The source of the contaminating fungus is possibly from the FF, and the culture medium does not regularly contain any antifungal agents, which is the cause of widespread fungal contamination in the culture of IVF. A lot of people are therefore opting for IVF to get a child. In vitro fertilization, assisted reproductive technologies (ART) includes extracorporeal fertilization using specific surgical procedures to support pregnant people. It is typically carried out when certain, less costly forms of reproduction struggle. Infertility is a global public health concern and accepted by Saudi society as a big issue. Many triggers can contribute to female infertility, such as ovulatory disorders, endometriosis, endocrine disorders, genetic factors, tubal factors, and pelvic inflammatory disease. Additionally, many factors in lifestyle, such as age, weight, obesity, smoking, environmental and other toxins, can affect overall health and lead to infertility. The embryo culture medium also contains antibacterial agents, but some species of bacteria may be resistant to these antibiotics and do not routinely produce any antifungal agents. The FF was not always sterile but contained a range of microorganisms that affected IVF results, and a broader sample of patients needed to be studied to further confirm our theory. Furthermore, identification of FF microbes in women with repeated failed IVF cycles will provide an opportunity to start antimicrobial therapy before the next pregnancy. The study aimed to provide a summary of the microorganisms and their effects on in vitro fertilization of human follicular fluid..

KEY WORDS: IVF, HUMAN, FOLLICULAR FLUID, MICROORGANISMS, IN VITRO, FERTILIZATION, OUTCOMES.

ARTICLE INFORMATION

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INTRODUCTION

Infertility is a global public health concern and is considered a major clinical problem today (Kumar & Singh, 2015). The prevalence of infertility measured by a systematic study of 277 health surveys from 1990 to 2010, in 190 countries. They found that this issue occurred in 48.5 million couples worldwide, 1.9% of women were unable to have their first child, which was identified as primary infertility, and 10.5% of women were unable to have another child after five years, which was represented as secondary infertility. This problem has been the most popular for some regions such as South Asia, North Africa, the Middle East, and Central Asia (Mascarenhas et al. 2012).In 2012, data were obtained and analyzed for 457 patients with infertility in the eastern region of Saudi Arabia. The prevalence of infertility was estimated to be 18.93 percent, higher than the prevalence in developing countries (3.5 percent to 16.7 percent) within one year (Al-Turki, 2015). The overall birth rate per woman in the world fell from 4,979 in 1960 to 2,432 in 2017, (Al-Turki, 2015; World Bank, 2017).

Infertility is now recognized as a major problem in Saudi society and a cause of concern. Many people, therefore, choose to have an assisted reproductive technology (ART) for having a child. One such technique is the treatment of IVF (Aoun & Moawed, 2012). In 2016, global data from 178 ART cycle centers in 15 Latin American countries were collected. They recorded 3931 IVF cycles, 1113 pregnancy rates, and 859 delivery rates at that time. Overall, the perinatal mortality rate was 8.2 percent in singletons, 19.31 percent in twins, and 63.2 percent in multiples of a high order. Compared to the previous year, the number of registered cycles increased by 14 percent, and the number of transferred embryos decreased with a decline in several births (Zegers-Hochschild et al., 2009). One of the most difficult situations when embryologists consider that after investing a lot of money and time, the embryo polluted. A second possible harmful effect when the microorganisms transmitted to the female reproductive tissue from the infected embryo culture media that can contribute to adverse pregnancy outcomes and several cases of embryo contamination are attributed to microorganisms from a follicular fluid (Pomeroy, 2010). In this fluid, the oocyte matures and the consistency of the oocyte influences embryo development and can be used as an embryo consistency biomarker (Chen et al., 2016).

Various micro-organisms may colonize the FF. These microorganisms are thought to have spread to the FF by hematogenic invasion from other body locations, such as the oral cavity and the respiratory tract (Pelzer et al., 2013a). Follicular fluid may also be infected by vaginal microorganisms when a large needle passed through the vagina into the ovary begins extracting the sample from the clinical embryologist. These microorganisms can produce many toxic substances, including endotoxins, alpha-hemolysin, Shiga-like, and other lipopolysaccharides and peptidoglycans, which may affect the medium of embryo culture and may result in DNA fragmentation of gametes, low-quality embryos and premature birth (Pomeroy 2010, Borges & Vireque, 2019).

Some microorganisms may be in low concentrations which affect the culture of the embryo but do not produce any obvious signs such as flocculants or embryo necrosis (Pomeroy, 2010). Traditionally, the embryo culture media contains antibiotics such as penicillin, streptomycin or gentamycin in an attempt to prevent the growth of pathogenic microorganisms while other forms of bacteria may be immune to these particular antibiotics and other microorganisms such as mycoplasms and anaerobic bacteria escape from the antibiotics placed on the culture of embryos (Pelzer & Allan, 2011 Borges &Vireque, 2019). Antimicrobials also have little inhibition of the potentially large number of bacteria in the culture medium (Moore et al. 2000). Also, in patients who received antibiotics before IVF cycles, the frequency of microbial infection after recovery declined from 0.4 percent to 0 percent (Gardner & Simón, 2017). Previous experiments associated FF microorganisms with declining or growing outcomes of IVF (Hamad et al., 2018; Ibadin & Osemwenkha, 2014; Pelzer et al. 2011; Pelzer et al. 2013a Kim et al. 2018).

The microbiome of semen has been studied mostly in connection with male infertility or prostatitis, Monteiro et al. (2018). Just a few microbial experiments of IVF have demonstrated that high-variety bacterial infection of the culture media in IVF induces injury or even destruction of oocytes and embryos produced. We aimed to determine and associate the prevalence and count of bacteria in IVF samples with clinical outcomes (Štšepetova et al., 2020). This research aims to obtain an overview of human follicular fluid microorganisms and their effects on in vitro fertilization outcomes.

The ovary and function: One coat of epithelium coats the ovary. The region pellucida is a sheet of glycoprotein encompassing the oocyte plasma membrane. The ovarian function is regulated by a nerve cell gonadotrophin hormone which sends its messages to the anterior pituitary gland to produce LH and FSH to grow follicles and ovarian steroid hormone output. In the center of each menstrual period, the ovaries produce one egg (oocyte), or occasionally two. This is called ovulation. The ovary has two main body reproduction features. (Bradford, 2017; Speroff & Fritz, 2005). The ovarian follicle comprises of an oocyte, enclosed by cell layers of granulosa, and an exterior basement membrane enclosed by additional layers of thecal cells and functioning together to synthesize the hormone to regulate the maturation of additional follicles. Once the reproductive hormones activate a certain egg for maturation, and the ovarian follicle goes through the following stages: primordial, main, secondary (pre-antral) and the final step is the pre-ovulatory follicle level. Mature follicles, known as Graafian follicles, can expand up to around 1.2 inches (30 millimeters) in diameter and the fluid that occupies the oocyte's cavity called follicular fluid. (Bradford, 2017; Fritz & Speroff, 2005).

Evaluation of oocyte quality: Before the IVF procedure evaluation of the infertile couple is important to achieve the best results and avoid complications. Women must then undergo blood tests for FSH, LH, prolactin, E2 (estrogen content in women's blood), and inhibit B rates, AMH, and Antral Follicle Count (AFC) with a high-quality trans-vaginal ultrasound scan that will provide the doctor with details on egg size and condition, ovarian reaction, and the appropriate way to implant the embryos. When the IVF process is completed with an elevated FSH level, the efficiency of ovarian stimulation is not enhanced, so women have a decreased cancelation risk. During the IVF process, too, most IVF practitioners undergo regular son hysterogram or hysteroscopy. The downside of hysteroscopy is that minor polyps or symptoms suggesting persistent endometritis are visualized (Gardner & Simón 2017). Pairs are screened for the presence of sexually transmitted infections (STIs) including C before IVF procedure. Trachomatis, that is N. *gonorrhea*, hepatitis B and C, human immunodeficiency virus (HIV), syphilis, and cytomegalo virus (CMV), but microbiological monitoring procedure for the IVF process is not done (Pelzer, 2011).

Upper genital tract (UGT): The women's reproductive organs' upward genital tract infection (UGTI) involving the endometrium, fallopian tubes, and ovaries is a prevalent disease among reproductive-age women. UGTI is typically triggered by an ascending infection of the form, where N is the most common cause. C or gonorrhea Trachomatis, but approximately 30 and 40% of cases are triggered by polymicrobial (Schiappacasse, 2014). Many experiments have reported that in the absence of symptomatic infection, micro-organisms colonize the female UGT (Pelzer et al. 2013a). Microbial contamination of UGT occurs due to LGT, especially while using a sample selection transcervical technique. The endometrial cultures lead obtained one or more microbes with *Lactobacillus spp*., M. Homa, G. Vaginalis, and Enterobacter spp. and another infection was induced by the tendency of such microbes to bind to human spermatozoa and then transmitted through the intrauterine area via the cervix. (Rampersaud et al. 2012).

The microorganisms which were extracted from PID cases were *S. horny, S. cohnii, Megaterium bacillus, Brevibacterium epidermidis, Francisella philomiragia, E. coli*, *Citrobacter freundii* (Okiki et al.2015). Many cervical swab experiments in people with bacterial vaginosis have shown that the first community of independent species is *E. coli* followed by Bacillus subtilis*, Proteus mirabilis* and *Actinomyces israelii* (Jabuk, 2014). Group B streptococci colonize 20-25% of pregnant women's maternal genital tract, which is a significant cause of neonatal illness which mortality (Stoll et al. 2011). Microorganisms inside the UGT, including those that contaminate the IVF culture method, can result in reduced oocyte content, embryo content (possibly due to fragmentation of oocyte DNA), And the failure of early infancy (Pelzer & Allan 2012). Patients with UGTI have an elevated chance of ectopic pregnancy and infertility correlated with damaged Fallopian tubes that develop after UGTI (Schiappacasse, 2014).

The placenta, fetal membranes, and cervical mucus function together during pregnancy to shield the baby from invasion by microorganisms. Inflammation of fetal membranes and placental chorion typically means that bacterial contamination is on the rise. Vaginal organisms are believed to initially invade the space between the tissues of the mother and the fetal membranes and afterward infect the amniotic fluid. (Rampersaud et al., 2012).Infertility described as women's inability to get pregnant during unsafe intercourse for 12 months (World Health Organization, 2016). It can be either primary infertility, which is a wait for a couple to conceive with no prior pregnancies or secondary infertility after one year or more, which is a pause for a couple who have produced children before (Anwar & Anwar, 2016).

This disorder will lead the infertile couple to depression and other psychiatric psychological disorders (Abolfotouh et al., 2013). There are two gonadotropin hormones produced in the pituitary gland, and the gonadotropin-releasing hormone (GnRH) [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] controls their secretion. The GnRH works on the pituitary gland to produce FSH and LH at the start of a new process. These hormones activate ovarian follicles and they grow them. Around 30-40 follicles begin to develop each month in response to FSH with the ability to release one single mature egg at fertilization ovulation (Anwar & Anwar, 2016).

The follicular fluid within the ovarian follicle: Follicular fluid is a substance that covers the ovum and develops from two outlets, the flow of the blood plasma portion connected with some thecal capillaries in the ovary's cortical area, and the secretory operation of granulosa and thecal cells. It includes many hormones including FSH, LH, GH, human chorionic gonadotropin (hCG), progesterone and estradiol (E2); enzymes; anticoagulants; electrolytes; reactive oxygen species; growth factors such as epidermal growth factor (EGF), EGF like growth factor (EGF), vascular endothelial growth factor (VEGF) and transforming growth factor-alpha (TGF-α); cytokines; antioxidants and metabolites and Multi-effecting fatty acids on ovarian development and oocyte maturation (Basuino& Silveira, 2016; Revelli et al., 2009). FF pH is stated to be between 7.2–7.3 (Swain, 2012). The Fallopian tube plays a crucial role in fertilization and early development of the fetus. FF is released into the peritoneal cavity at ovulation and inserted into the Fallopian tube to influence reproductive parameters and promote embryo cleavage during IVF (Lyons et al., 2005).

The follicular fluid has an essential role in antral follicle contact between cells when transporting nutrients through the oocyte. FF is also a core component of the effectiveness of natural fertilization present at any point in the design process (Basuino & Silveira, 2016; Revelli et al., 2009). Hormones, gonadotropins play a

significant role in the secretion of several substances that influence oocyte production and maturation by granulosa cells. The elevated FSH, hCG and LH rates have been related to oocyte maturation and fertilization (Revelli et al., 2009). In comparison, PCOS patients, low levels of FF components such as testosterone, E2, progesterone, and β-hCG, can have a detrimental effect on oocyte production and fertilization rates (Basuino & Silveira, 2016).

Growth hormone (GH), Granulosa cells boost the FSH and develop FSH and LH receptors in certain cells. Growth hormone production also happens in the follicle; thus, it may interact with gonadotropins that increase the amount of estrogen contributing to improved oocytes. There is no clear correlation between GH levels intrafollicular and the rates of pregnancy.Prolactin (PRL): Several types of research found a link between high PRL with fertilization and positive pregnancy, but this was not supported by other tests. Hence FF PRL is not considered a strong sign of oocyte quality at present, (Revelli et al. 2009). Estrogens, progesterone, and androgens: Several types of research have shown that the strong FF estrogens ratio correlates with oocyte maturation may contribute to a higher risk of pregnancy but not supported by others.

Optimal progesterone exposure has beneficial effects on oocyte characteristics although inappropriate exposure contributes to a decrease in oocyte production. In comparison, elevated androgen rates (testosterone) associated with lower-quality oocytes. Inhibin: Granulosa cells manufacture inhibin and are classified into two forms (inhibin A, and B). Throughout the FF, inhibin A improves throughout women with endometriosis during the follicular period, and higher, while inhibin B reduces. Inhibin B in FF correlated with the number of oocytes retrieved, but not with the result of the IVF, may, therefore, be viewed as a symbol of ovarian reaction but not of oocyte efficiency (Revelli et al., 2009). Anti-Mullerian hormone (AMH): There is already an inconsistent association between oocyte production and AMH rates. Some studies noticed that rates of AMH were associated with oocyte production, and others showed that rates of AMH were linked inversely with oocyte maturation (Revelli et al., 2009).

The normal genital tract flora and opportunistic pathogens: Lower genital tract, *Lactobacillus* species predominate the normal lower genital tract flora (LGT) for most healthy women. The species most common include *L. crispato, L. down, L. jensenii* & *L. gasseri gasseri*. Their capacity can provide protective functions through the production of lactic acid to preserve an acid environment, hydrogen peroxide, and other substances that prevent pathogenic microorganisms from overgrowth. When the vagina lacks lactobacilli, other lactic acid bacteria in the vagina, including the species Atopobium, Megasphaera and Leptotrichia (Lamont et al., 2011; Witkinet al., 2007).

In addition, any overgrowth disturbance of the usual vaginal flora, especially anaerobic species *Mycoplasma hominins, Gardnerella vaginalis, Bacteroides* and *Mobiluncus* can contribute to bacterial vaginosis (BV). Gray or yellow blood, fishy odor, and stomach pain are the most frequent signs of BV. Up to half of the people are asymptomatic. The clinical treatment describes this variation with low pH>4.5, fishy odor on the introduction of 10 percent KOH, and the presence of hint cells under microscopic vaginal smear inspection (Morris et al., 2001).BV incidence has been associated with various gynecological disorders and pregnancy problems such as pelvic inflammatory disorder (PID), miscarriage, endometriosis, and preterm delivery (Lamont et al., 2011; Nelson & Macones, 2002). Many types of research before and after the ART procedure indicated that the prevalence of BV among infertile women was 4.2-36 percent (Morris et al., 2001; Spandorfer et al., 2001; Wilson et al., 2002). Opportunistic pathogens like *Staphylococci*, *Enterococci, Enterobacteria, Candida, Peptostreptococci, Peptococci fungi*, and Gram-negative anaerobic bacteria were the most widespread microorganisms found in women's vaginal discharge.(Aleshkin et al., 2006).

An anaerobic overgrowth of the regular flora of aerobic microbes including *Escherichia coli*, group *B Streptococci, Enterococci*, and *Staphylococcus aureus* may disrupt the abnormal vaginal flora. Pregnancy risks are the most significant contributing factors for a vaginal infection, such as early membrane breakup, early delivery, and perinatal infection. *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Haemophilus influenza*, and group B *Streptococci* are the forms of pathogenic bacteria that may be linked with such complications (Donati et al., 2010).Additionally, Candida spp. It is a natural commensally yeast in the female genital tract and may sometimes cause diseases varying from mild to extremes, such as vaginitis, cervicitis, and persistent vulvo vaginal candidiasis (Pellati et al., 2008). Contamination with candida Albicans happens predominantly (80 to 90 percent) in confirmed patients, whereas contamination with other pathogens happens less often, including *C. glabrata* and *C. tropicalis* (Soong & Einarson, 2009).

Microorganisms within Human Follicular Fluid Effects on IVF: In both normal and IVF pregnancies, the existence of opportunistic pathogens in the lower female reproductive tract was correlated with adverse outcomes of pregnancy (McClure and Goldenberg, 2009). Discrepancies in women with colonized and infected follicular fluid were found (Pelzer et al., 2011). Also, multiple reports have reported that, in the absence of asymptomatic infection, microorganisms often and transiently colonize the female upper genital tract (Viniker, 1999). Studies of microorganisms and human follicular fluid were performed predominantly in women engaging in IVF cycles owing to the complexity of the procedures needed to acquire this specimen (Pelzer et al., 2011). Cottell et al., (1996) in their report, examined the impact of microorganisms from the IVF culture method as a whole by pooling the findings obtained for each test form (follicular fluid, oocyte extraction needle wash, semen and culture media) and finding correlations between

these tests and IVF outcomes and concluding that no adverse effects occurred (Cottell et al., 1996, Pelzer et al., 2013).

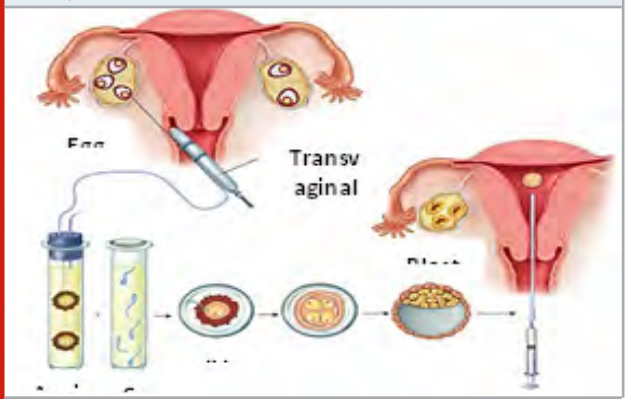
The IVF is not possible in a sterile setting. During semen therapy, the frequency and concentrations of bacteria reduced during the IVF process. The prevalence of Bacilli (Lactobacillus genera) groups in raw semen and IVF culture media, Clostridia in washed sperm, and *Bacteroidia* in incubated sperm samples was shown. *Staphylococcus sp* has an appearance. *Alphaproteo bacteria* and health measures such as semen and embryo production are correlated with this. Potential studies will also concentrate on strategies to help reduce the harmful effects of these microorganisms on the development of IVF embryos and to help deter IVF failure (Monteiro et al., 2018 and Štšepetova, et al., 2020).

Microorganisms and IVF outcomes: Techniques for in vitro fertilization are vulnerable to infection on several levels. Oocyte infection or the developing embryo can occur during IVF procedures. Past research also indicated a link between FF-isolated micro-organisms and IVF outcomes. Kim et al. (2018) analyzed collections of vaginal swab and FF from infertile women completing IVF cycles at the time of ovum processing. The isolated bacterial species is coagulase-negative *Staphylococci*, *Streptococcus* agalactiae, *E. coli, Kristina kocuria, E. fecalis, pneumonic Klebsiella,* and *S. aurora.* They stated that FF is not sterile but that FF microorganisms do not have any major adverse effects on IVF tests. Studies with a vaginal swab and FF tests in people that have had IVF periods.

They showed that 45.7 percent of FF samples had bacterial organisms, including *Lactobacillus spp., Staphylococcus spp., Streptococcus spp., Propionibacterium spp., Actinomyces spp.,* and *Bifidobacterium spp.* Haahr et al. (2016) analyzed vaginal swabs for specific *Lactobacillus spp.*, *Gardnerella vaginalis*, and extracted *Atopobium vaginae* from women in IVF periods. They revealed the *G. vaginaliz, A. vaginae* were BV associated. *Lactobacillus crispatus*, *L. jensenii y, L. gasseri* were correlated with natural microbiota and the presence of unhealthy vaginal microbes (AVM) in IVF patients could have a detrimental impact on fertility levels and other reproductive outcomes. If there was a significant association between AVM and the results of abortion, patients should be tested and monitored with AVM until the IVF treatment begins.

Pelzer et al. (2013a) isolated FF and vaginal swabs for microorganism detections from women undergoing IVF procedure with various infertility causes at the time of oocyte retrieval. They estimated that the prevalence of FF micro-organisms was 99 percent; infected 71 percent of FF and colonized 29 percent. *Propionibacterium spp., Streptococcus spp., Actinomyces spp., Staphylococcus spp.,* and *Bifidobacterium spp.* have been correlated with negative IVF results whereas *Lactobacillus spp.* is present. Heightened embryo transfer levels were consistent with this. Pelzer et al. (2013b) investigated the DNA heterogeneity inside FF of non-fertilized mouse oocytes incubated in vitro from women undergoing IVF procedures. To detect the existence of bacteria, each sample of FF was cultured. They displayed other bacterial forms including *Streptococcus anginosus*, *Peptoniphilus spp.*, *Lactobacillus gasseri*, *E. fecalis*, and acnes containing *Propionibacterium*. Much fragmentation of DNA occurred with a large dose of *P. acnes or L. gasseri.* No fragmentation of DNA with a low dose of L was observed. *L. gasseri* and *E. fecalis* as shown in Fig 1. They concluded that FF microorganisms can contribute to oocytes of poor quality that lead to reduced IVF outcomes.

Figure 1: In vitro fertilization procedure (Hoffman et al., 2016).



Association between anti-chlamydial immunity and IVF outcome: Chlamydial inflammation is one of the main sources of occlusion of the fallopian tube. The latest studies have shown that serum anti-chlamydial antibodies are present in almost every second where individual receiving in vitro fertilization (IVF) therapy for tubal infertility (de Barbeyrac Papaxanthos-Roche, 2006 and Muller et al., 2015). Several reports have examined the connection between the immune reaction to the *Chlamydia trachomatis* and the result of IVF. Neuer et al.(1997) reported a decrease in pregnancy levels for anti-chlamydial IgA in follicular fluid in women with IVF positive. Liccardi et al. (1992) identified a correlation between serum positivity of the chlamydial antibody and spontaneous abortion. In comparison, a comparable number of publications, Gaudoin et al. (1999) and Muller et al. (2015) reported no association between anti-chlamydial immunity and IVF loss. Data are present in various research on potential pathways involved in the production of infertility following chlamydial infection, although it remains contentious. Hence, our study's key objective was to evaluate the effects of microorganisms on in vitro fertilization of human follicular fluid.

Endocrine disorders: The pituitary gland was split into two lobes, the pituitary posterior (neurohypophysis) and the pituitary anterior (adenohypophysis). The anterior pituitary controls the release of the thyroid-stimulating hormone (TSH), the adrenocorticotropic hormone (ACTH), the thyrotropin, the corticotrophic, and the gonadotrophic cells respectively. The anterior pituitary also secretes

growth hormone (GH) and prolactin, respectively, from the somatotroph and lactotroph cells. Several factors, such as genetic abnormalities, diet, drugs, inflammatory processes, and certain tumors may affect the pituitary role. The abnormality or deficiency of the pituitary gland, which can contribute to excessive prolactin (hyperprolactinemia) will inhibit ovulation. Thyroid conditions(hyperthyroidism and hypothyroidism) can also interfere with ovaries that can trigger ovulation delay (Pauli and Kallen 2011, Anwar & Anwar 2016).

Premature ovarian failure: In people less than 40 years old, this is a disease of discontinues usual ovarian activity. It is normally triggered by an allergic reaction or early depletion of ovary eggs probably induced by genetic factors or chemotherapy. In women less than 40 years old, natural ovarian activity stops. The most frequent signs include prolonged or missing cycles, hot flashes, and sweating at night. This disorder is a disease of elevated FSH rates, low estradiol rates, and reduced hormone levels (Unuane et al. 2011).Endometriosis, a common debilitating condition that affects 10 percent of women of reproductive age, triggering pelvic pain, and infertility. The incidence of endometriosis in women with infertility rose by up to 50 percent. It is known as endometrial glands and stroma developing outside the uterus on other pelvic organs such as ovaries or Fallopian tubes. This tissue has no way to leave the body, it gets stuck and cysts grow. The surgical removal of it may induce scarring, which can obstruct Fallopian tubes and prevent the joining of an egg and sperm. Compared to women with unexplained or tubal infertility, the existence of endometriosis may adversely affect spontaneous pregnancy and IVF outcomes, (Khine et al. 2016).

Genetic factors: It is a genetic disorder influencing the role of the ovaries. Girls with Turner's syndrome have distinctive facial characteristics and reproductive defects of the X chromosomes because of incomplete or anomalies. Any of the girls impacted was unable to reproduce because of an ovarian defect. Patients with Turner's syndrome get an appropriate level of conception after oocyte donation. A high pregnancy prevalence associated with hypertensive disorders that can contribute to restriction of premature birth and intrauterine development (Bodri et al., 2005). It is the absence of an apparent cause of infertility that occurs in about 15–30 percent of infertile couples. It can only be diagnosed after the male and female partners have completed a fertility assessment (Schattman et al., 2016).

Lifestyle influences are modifiable habits and activities which may influence physical health and lead to infertility. Many factors in lifestyle such as age, nutrition, obesity, exercise, smoking, consumption of caffeine, psychological stress, pollutants in the environment, and others can have an impact on fertility. There is clear proof that sex, weight, and smoking influence general wellbeing and detrimental impact on sexual health, (Homan et al. 2007).These considerations are further explored in-depth below: As women enter the age of 35, their fertility declines, rendering pregnancy more complicated due to the reduction of both oocyte and follicle pool quantity and consistency. When women are younger than 30 years of age, the chances of conception can reach 71%; when they are older than 36, they can only be 41%. With age increases, women are at increased risk of fertility-influencing conditions such as uterine fibroids and endometriosis (Homan et al., 2007; Velde & Pearsom, 2002 Hoffman et al 2016).

Obesity is associated with higher plasma and FF leptin production. Leptin was also found to inhibit the production of LH by the granulosa cells which stimulated estradiol production. Such effects can explain in part the decreased reproductive success of women with overweight (Metwally et al., 2007a). A background of infection with the genital tract All aspects of the female reproductive system may be affected by infectious agents and impair female fertility. Infectious diseases may impact multiple reproductive tract sites and include damage to the cervical, tubal, and peritoneal. The most popular female fertility-related micro-organisms are C. trachomatis and *N. Gonorrhoeae*, which considered being the most significant cause of tubal lacerations and obstruction, PID, and adhesions. Microorganisms which are associated with bacterial vaginosis can reach the genital tract in different ways and may result in infertility to the tubal factor (Pellati et al., 2008). In smoking, miscarriage rates in both natural and assisted conception cycles, ectopic pregnancy, early menopause, and infertility are the reproductive risks associated with smoking. Cigarette smoke substances such as nicotine and carbon dioxide and cyanide may influence the follicular microenvironment, modify the hormone rates, increase genetic defects, placental insufficiency, and lack of reproductive ability. In female smokers' FF, cotinine and cadmium have been found, and this can impact the forming follicle (Homan et al 2007, Hoffman et al., 2016).

Reactive Oxygen Species and antioxidant factors: Some studies found a positive correlation between levels of FF, Reactive Oxygen Species (ROS) and maturation of the oocytes. The women who were pregnant with IVF had higher rates of ROS in the FF relative to non-pregnant people. On the other hand, the etiology of defective embryo development is associated with oxidative stress. The presence of antioxidants and reactive oxygen species in the FF helps the meiosis cycle to take place (Revelli et al., 2009). Cytokines and growth factors, cytokines are required for ovarian activity, modulating ovarian steroid hormone production, and embryonic growth. Many studies have examined the association between FF cytokines and ART outcomes: these researchers found that cytokines and VEGF were associated with higher fertilization levels, positive embryo transfer, and clinical pregnancy, whereas interleukin (IL) -12, VEGF and IL-15 were associated with low fertilization and aborted conception (Pelzer et al., 2011). Other contents, the peritoneal fluid associated with FF is an essential factor for the ovulation process, gametes transfer

and preservation, and oocyte-sperm involvement in environmental development. In the FF lipoprotein acts as a medium for progesterone production. High-Density Lipoprotein (HDL) also lets the oocyte and embryo grows well (Basuino & Silveira, 2016).

CONCLUSION

There are elevated antibody titers against *Chlamydia trachomatis* found in up to 70 percent of people with tubal factor infertility (TFI). Determining the influence of past chlamydial agents. Longitudinal retrospective research on the effects of an infection with an IVF procedure was conducted. Detection of an anti-chlamydial antibody is not linked to the formation of oocytes, the growth of eggs, the pregnancy, and the birth of life stages. Thus, past chlamydial infection in TFI patients is correlated to ovarian stimulation and missed risk of abortion with decreased IVF success. The FF was not sterile but had a range of micro-organisms that influenced IVF results, so a broader population of patients had to be tested to further support our hypothesis. Indeed, it would provide an incentive to begin antimicrobial therapy before the next birth to recognize FF bacteria in individuals with multiple missed IVF cycles.

Conflict of interest: The authors declare no conflicts of interest.

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Nutritional Communication

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Effect of Incorporation of Roasted *Ocimum basilicum* L. Seeds on the *In vitro* Glycemic Index of Steamed Rice Cake

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ABSTRACT

The most populous South Indian staple food, steamed rice cake or idli is high in glycemic index. Still, the consumption frequency of idli is very high, at least twice a day, even by people with diabetes. *O.basilicum*, also called as basil or sabja seeds are effective in controlling diabetes by slowing down the conversion of carbohydrates to glucose. It is unfortunate that the basil seeds are not commonly used by the Indian community. As a primitive measure, the role of unexploited basil seeds in controlling diabetes by supplementing into the frequently consumed food product, steamed rice cake or idli has been determined in this study.This study aims to study the efficacy of incorporation of roasted *O.basilicum* seeds on the in vitro glycemic index values of steamed rice cake. Roasted Ocimum basilicum seeds in proportions of 5% (RV1), 10% (RV2), 15% (RV3) and 20% (RV4) of the black gram have been incorporated into the traditional high glycemic South Indian food, steamed rice cake. All the variations of roasted Ocimum basilicum seed incorporated steamed rice cakes were subjected to carbohydrate profile evaluation and estimation of in vitro glycaemic index. The results depicted that the composition of detected parameters of the roasted *O.basilicum* seed incorporated steamed rice cake showed significant difference ($p<0.05$) in all the variations, on comparison with control. As the proportion of *O.basilicum* seeds increased the total carbohydrate and sugar values showed a decline, whereas the cellulose, hemicellulose and resistant starch values increased. The mean in vitro glycemic index of the control steamed rice cake was 68.67 ± 0.02, while the mean estimated glycemic index value of RV1, RV2, RV3 and RV4 were 56.73 ± 0.01, 54.78 ± 0.02, 52.63± 0.02 and 51.66 ± 0.01 respectively. Thus, the roasted seeds of *O. basilicum* can be effectively used in steamed rice cake preparation as a means to lower glycemic index at an affordable cost.

KEY WORDS: DIABETES MELLITUS, GLYCEMIC INDEX, IDLI, OCIMUM BASILICUM, ROASTING.

INTRODUCTION

The diabetes indices of global population is in rise alarmingly every other day. In each part of the universe several studies and researches are being carried out to find solutions to keep diabetes at bay. Indeed, meek measures like lifestyle changes and simple and economical dietary modification is the need of the hour to control diabetes and pre diabetes. Rice is a major food source in the dietary pattern of the South Indians. Though rice is a prime energy provider, and has many vital components like B vitamins in it, the lack of fibre and high glycemic index (GI=73) makes rice a foe to people with sedentary life style and diabetes. Glycemic index is considered as an important indicator of glycemic response (Jenkins et al, 1981). The rate at which the blood sugar levels raise after ingestion of a particular food in an observed period of time in comparison with the controls like glucose or

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white bread is termed as Glycemic Index. The lower the GI of a food the better it is for diabetics to consume.

Foods with GI value less than 55 can be categorized as low GI foods, while with GI values between 56 and 69 are termed as medium GI foods and foods with GI value more than 70 are grouped as high GI foods. There are several studies which have been undertaken to reduce the glycemic index of rice incorporated food products, either by adopting change in the rice processing methods or addition of ingredients which are high in fibre or fat to counter act the impact of glycemic response of rice. One such example is optimizing cooking temperature and the ratio of water to rice reduced in vitro starch digestibility and GI of instant rice, (Ritudomphol et al. 2019).

Similarly, Steamed rice cake or idli, a cereal legume based spongy and soft textured fermented food consumed by South Indians (Sonawane etal, 2019) as their diurnal diet which is high in GI, has been modified by various measures. The nutritive value of idli was enhanced by addition of sorghum and pearl millet (Nazni et al, 2010). Amarant grain flour had been incorporated to develop value added idli (Nazni et al, 2014). Heat treatment of rice was chosen for the preparation of low-GI Idli, (Chelliah et al, 2019). Oats flour and guargum flour incorporation reduced the GI of idli (Giri, 2017). Research study on addition of jowar to idli has shown a low glycemic response compared to rice rawa idli, (Jahan, 2018). Leaves and seeds of *Ocimum* species due to their medicinal properties is in use in traditional medicine for treating various ailments. Basil leaves lowered blood glucose levels and advanced glycation end products in diabetic rats (Widjaja et al, 2019).

The presence of polyphenols and flavonoids showcases *O. basilicum* seeds as a vital part of the daily food regime (Sestili et. al, 2018). The aqueous extract *O.basilicum* or basil or sabja seeds either by antioxidant or by α-glucosidase and α-amylase inhibiting activities, offered positive benefits to control diabetes (El-Beshbishy et al, 2012). Renal damage induced by diabetes was reverted by *O. basilicum* in albino rats and has been proven biochemically and histo pathologically, (Almalki 2019). In this context, this study has aimed at studying the effect of roasted *O.basilicum* or basil or sabja seeds on the *in vitro* glycemic index values of steamed rice cake.

MATERIAL AND METHODS

Procurement and Processing of Raw Material: The raw materials required for the study such as parboiled rice, dehulled black gram and basil or sabja seeds (*Ocimum basilicum* L.) were purchased from the local market of Salem district, Tamilnadu. The ingredients rice and black gram were hand sorted, to make sure that only quality grains are used and the impurities were removed by washing with water. The basil seeds were hand sorted and sieved to ensure quality. The seeds were dry roasted at 1150 C for 6-8 minutes and cooled, roasting beyond this temperature charred the seeds, while roasting lesser than 1150 C did not bring about changes in the colour and aroma. Roasting was done as it improves antioxidant capacity and oxidative stability, (Durmaz et al, 2010).

Table 1. Ingredients in the Preparation of Control and Roasted *O.basilicum* Seeds Incorporated Steamed rice Cake

Ingredients	Control Steamed Rice Cake	Level of Incorporation			
		O.basilicum Seeds Incorporated Steamed rice Cake			
		RV1	RV2	RV3	RV4
Rice (gms)	100	100	100	100	100
Black gram (gms)	100	23.75	22.5	21.25	20
Roasted *O.basilicum* seeds (gms)	-	1.25	2.5	3.75	5

RV1—Roasted Variation 1, RV2—Roasted Variation 2, RV3—Roasted Variation 3, RV4—Roasted Variation 4

Formulation of Roasted *Ocimum basilicum* L. Seed Incorporated Steamed Rice Cake: The cleaned parboiled rice and dehulled black gram were soaked for 5 hrs in water at room temperature separately in the ratio of 4:1 (Ghosh et al, 2011), for control and in different proportions for respective variations of steamed rice cakes as shown in Table 1 and ground to batter. The batter was allowed to ferment for 7 hours (Nagarjuna et al. 2000). The batter was beaten well and roasted *O. basilicum* seeds at 5, 10, 15, and 20% levels of black gram were incorporated into the respective proportions of batter and was allowed to stand for a period of 15 minutes (Samateh et al, 2018) for the seeds to gel. The batter was poured in an idli steamer and steamed till doneness which approximated to 5 to 8 minutes. The roasted *O. basilicum* seed incorporated steamed rice cake is done. Simultaneously the control steamed rice cake was prepared by following the same procedure without the addition of basil seeds.

Determination of Carbohydrate profile: The developed variations of steamed rice cake and control were subjected to estimate the carbohydrate profile indices namely total carbohydrate, sugars, cellulose, hemicellulose and

resistant starch. Carbohydrate and sugar content was assessed by IS 1656 and IS 6287 procedures respectively, while cellulose, hemicellulose and resistant starch in steamed rice cake samples were analyzed using standard procedure by Mathews et.al (1993) and AOAC, (2002).

Estimation of In Vitro Glycemic Index: Starch Hydrolysis percentage, (C∞ %) corresponds to the concentration at equilibrium (t180) and k is the kinetic constant. The hydrolysis index (HI) was derived by dividing the area under the hydrolysis curve of each variation by the corresponding area of a reference sample (glucose). The estimated glycemic index (EGI) was calculated using the equation, EGI =39.71 + (0.549HI).

RESULTS AND DISCUSSION

The results and discussion pertaining to the study, Effect of incorporation of roasted *Ocimum basilicum* L. seeds on the *in vitro* glycemic index of steamed rice cake are presented below.

Table 2. Carbohydrate Profile of Roasted *Ocimum basilicum* L. Seed Incorporated Steamed Rice Cake

Variations	Carbohydrate (%)	Sugar (%)	Cellulose (%)	Hemicellulose (%)	Resistant Starch (%)
Control	21.49±0.37^{a}	0.22±0.02^{a}	1.02±0.47^{c}	1.04±0.054^{c}	0.67±0.64^{c}
RV1	19.72±0.17^{b}	0.18±0.04^{c}	1.73±0.081^{b}	1.89±0.110^{b}	0.84±0.030^{b}
RV2	18.10±0.12^{c}	0.19±0.03^{b}	1.54±0.090^{b}	2.11±0.035^{a}	0.86±0.010^{b}
RV3	18.78±0.22^{c}	0.13±0.03^{e}	2.10±0.020^{a}	2.63±0.081^{a}	0.91±0.030^{a}
RV4	14.79±0.31^{d}	0.14±0.03^{d}	2.14±0.056^{a}	2.66±0.122^{a}	0.98±0.045^{a}

Each value in the table are represented as Mean ± SD. Means with same superscript are not significantly different using Duncan's Multiple Range Test (P < 0.05).

Carbohydrate Profile of Roasted *Ocimum basilicum* L. Seed Incorporated Steamed Rice Cake: The most common and abundant forms of carbohydrates are sugars, fibers, and starches (McMacken et al, 2017). Table 2 depicts the carbohydrate profile indices of the developed variations of roasted *O. basilicum* seeds incorporated steamed rice cake and control steamed rice cake. The mean carbohydrate value in the control steamed rice cake was 21.49 and the carbohydrate percentage has decreased gradually as the incorporation quantity of basil seeds increased (RV4<RV3<RV2<RV1). It is to be noted that the sugar composition is inversely proportion to roasted *O. basilicum* seed composition. The parameters viz. resistant starch and the non-starch polysaccharides, like cellulose and hemicellulose are undigested, but are the major components of dietary fiber that are fermented by the colon microbiota to produce short chain fatty acids (Lovegrove et al.2017). The values of cellulose, hemicellulose and resistant starch have amplified when compared with standard proportionally and is statistically significant at (P < 0.05).

Table 3. Percentage of Starch Hydrolysis, Hydrolysis Index and Estimated Glycemic Index of Roasted *Ocimum basilicum* L. Seeds Incorporated Steamed Rice Cake during an In vitro Ingestion Process

Sample	C∞ (%)	K	Calculated HI (%)	EGI (%)
Control	59.13 ± 0.03^{e}	0.0395 ± 0.00^{a}	52.75 ± 0.03^{e}	68.67 ± 0.02^{e}
RV1	42.77 ± 0.02^{d}	0.4998 ± 0.00^{b}	31.00 ± 0.02^{d}	56.73 ± 0.01^{d}
RV2	38.75 ± 0.04^{c}	0.9854 ± 0.00^{c}	27.44 ± 0.03^{c}	54.78 ± 0.02^{c}
RV3	33.36 ± 0.04^{b}	0.9840 ± 0.00^{d}	23.53 ± 0.03^{b}	52.63 ± 0.02^{b}
RV4	30.84 ± 0.03^{a}	0.9056 ± 0.00^{e}	21.77 ± 0.02^{a}	51.66 ± 0.01^{a}

Each value in the table are represented as Mean ± SD. Means with same superscript are not significantly different using Duncan's Multiple Range Test (P < 0.05). C∞, equilibrium starch hydrolysis percentage; k, kinetic constant, HI, hydrolysis index and EGI, Estimated glycemic index.

It is also evident that the variation 4 with 20% of the basil seeds possess low sugar, high cellulose, hemicellulose and resistant starch compared to the control and the rest of the variations of steamed rice cake. The dietary

fibres, short chain fatty acids and microbiota improve the immune cell functioning and also regulates blood sugar levels (Chang, 2018). Moreover, the study of Praznik et al, (2016) states that the polysaccharides in basil seed brands them to be considered as an excellent prebiotic medium inducing the growth of lactobacilli strains, the probiotics that play a key role in controlling blood sugar levels. It is also established that the Ocimum basilicum L. seed with the manifestation of resistant starch, promises as a functional ingredient (Fuentes-Zaragoza et al, 2010). Hence, the variation 4, steamed rice cake with 20% of roasted *O. basilicum* seeds can be subjected to further studies for glycemic index and impact on diabetic profile.

In vitro Glycemic Index of Roasted *Ocimum basilicum* L. Seeds Incorporated Steamed Rice Cake: An *in vitro* starch hydrolysis method was carried out in this study, to simulate the in vivo situation of carbohydrate digestion characteristics and to estimate the metabolic glycaemic response.

Table 3 indicates the hydrolysis index (HI) and assessed GI values of the control and *O. basilicum* seeds added steamed rice cake variations. At a higher *O.basilicum* seed substitution level, there was significantly lower ($p < 0.05$) in vitro starch hydrolysis. Overall, with an increase in the proportions of the *O. basilicum* seeds, from 5% to 20%, the HI values declined from 31% to 21.77%, with drop in estimated glycemic index values from 56.73 to 51.66 which were statistically significant at $p < 0.05$. A similar anti- hyperglycemic effect was studied by ingesting aqueous extract of *Ocimum basilicum* seeds in Streptozotocin induced diabetic rats (Chaudhary et. al 2016), Goñi et al. (1997) have also proved that in vitro methods of GI estimation correlate well with the in vivo method.

CONCLUSION

O. basilicum seeds are food materials, with pharmaceutical properties, which have been used to dispel many diseases from ancient times. Considerable amount of mucilage appears around the basil seeds when they soak in water, which is a rich source of hydrocolloid with outstanding functional properties. The mucilaginous and anti-diabetic properties of the *O.basilicum* seeds had been utilized in the steamed rice cake preparation. From the study conducted, in vitro starch hydrolysis of steamed rice cake variations was significantly affected ($p < 0.05$) by the amount of *O. basilicum* seeds added in the steamed rice cake formulation. Resistant starch content increased significantly ($p < 0.05$) in 15% and 20% steamed rice cake variations, compared with the control. Hence, in vitro starch hydrolysis is closely related to the amount of resistant starch present in steamed rice cake variations. Also, resistant starch content is inversely related with hydrolysis index value, which resulted in lower estimated glycemic index values at higher added *O. basilicum* seed levels. It can be concluded that the roasted *O.basilicum* seeds incorporated rice cakes can be effectively utilised as an anti-diabetic food and the micro vascular and macro vascular complications allied with diabetes can also be prevented to a larger extent.

Conflict of Interest: The authors declare no conflict of interest

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Preparation of Bimetallic and Trimetallic Nanomaterials and their Role in Waste Water Treatment: A Review

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ABSTRACT

Multimetallic nanoparticles (NPs) have extraordinary properties and therefore, drew the attention regarding their synthesis and applications in the form of bi and tri metallic nanoparticles. Bimetallic (BNPs) and trimetallic nanoparticles (TNPs) are gaining enormous attention than that of monometallic nanoparticles. Both NPs can be synthesized by different methods such as microwave, selective catalytic reduction, micro-emulsion, co-precipitation and hydrothermal etc. Using physical and chemical methods have more disadvantages such as production of toxic byproduct, use of excess energy and additional use of stabilizer. In addition, nanocomposites of bimetallic and trimetallic can be synthesized with inorganic and organic compounds such as: carbon, graphene, gelatin, cellulose, starch, chitosan, alginate, etc. The combination of two or more phases in these nanoscale materials provide them high surface area to volume ratio and possess higher degree of porosity that help in enhancing their adsorption and reusability found more helpful in removing the toxic pollutants from the environment. Further these nanomaterials can also be fabricated in such a way that reduces the electron hole recombination, which induces synergetic effect between the constituent moieties that help in the degradation of pollutants. For instance the synthesis of trimetallic nanostructures with defined design along with the required morphology as well as mesoporous and magnetic characteristics have shown their versatile properties find applications in many industries such as conducting magnetic inks, memory devices, catalysis, bio-medical and especially in water treatment. Although, to obtain the nanoparticles with desired morphology and size is relatively difficult, which involves expensive non-eco-friendly reagents. In this review, we discussed in detail about the synthesis and role of Bimetallic and Trimetallic NPs as an adsorbent.

KEY WORDS: NANOPARTICLES, BIMETALLIC, TRIMETALLIC, WASTE WATER TREATMENT AND ADSORPTION.

INTRODUCTION

The significance of nanotechnology and nanoscience has been mainly associated with fabrication, characterization and applications of nanoscale materials in the form of nanorods, nanotubes, nanoparticles, nanosheets and nanoporous structures. They have been developed through the association of atomic and molecular clusters (Pitkethly 2004). Nanoparticles (NPs) may be defined as they possess at least one of the dimensions of nanosize. They are lying in the nanoregime i.e. in between 1nm to 100nm range (Jain et al., 2006). They act as the bridge between the bulk and their atomic structures (Luo et al.,2006). Bulk materials exhibit regular physiochemical properties regardless of their size.

However, when the same materials acquire the nano-size, they start to show highly enhanced useful

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physico-chemical, electronic, electrical, magnetic, optical, catalytic properties etc. as compared to their conventional counterparts (Jiang et al., 2008).This can be attributed to the high surface area to volume ratio (Schrand et al., 2010). Because of these characteristic properties NPs have shown tremendous potential for their applications in the field of engineering, environmental, biological sciences as well as in biotechnology (Mishra et al.,2015). NPs also play a pivotal role in many catalytic and biochemical processes (Guildford et al. 2009, Wang et al. 2019). The concept of nanoparticle and their application in biological systems also has advantages over other materials because their size is very much close to the size of cellular components (Yang et al. 2019). For instance, the size of a DNA molecule is about 2.5 nm, thickness of biological membrane is 6 nm and a protein is approximately 50 nm wide (Liu et al. 2018). Besides this, NPs plays significant role to analyze toxic dye removal from industrial wastewater (Leite et al. 2018, Sonkusare et al. 2020). The NPs classification, synthetic procedures and their applications in dye removal and cancer treatment are discussed in the proceeding units.

Classification of NPs: Based on structure, morphology and size, the NPs of various shapes are presented in fig 1 a-b and further discussed as follows.

Based on dimensions: Based on their dimension and aspect ratio, NPs can be classified into four classes. The general characteristics of these nanoparticles are discussed in Table1.1, covering their definitions and area of applications, while their two and three dimensional structures are given in Fig. 2.

Figure 1a: Comparison of size in nanoparticles, (Brar et al., 2010)

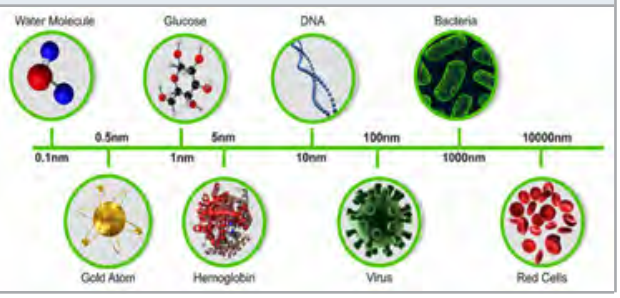


Figure 1b: Examples of nanoparticles based on constituents and structures, (Silva et al., 2019)

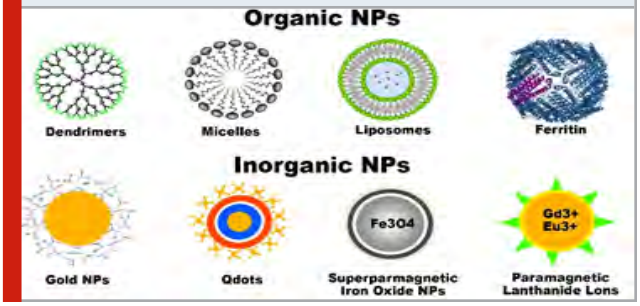
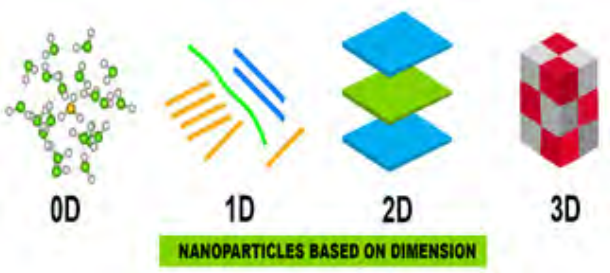


Table 1.1. Types of NPs, their definition along with examples and their uses.

Types of NPs	Definition	Examples	Uses	Ref
Zero Dimension (0D)	NPs having each of three dimensions limited in the nanoscale.	Fullerenes Composite NPs Core Shell NPs	In various biomedical applications	(Barnakov et al. 2019)
One Dimension (1D)	NPs have two dimensions in the nanoscale.	Nanotubes Nanorods	Energy harvesting, Storage efficiency	(Linfeng et al. 2019)
Two dimensional (2D)	NPs having one dimension in nanoscale	Nano films, Nanosheets	Biochemical sensors, Catalysis	(Yola et al. 2018)
Three dimensional (3D)	NPs which no dimension are confined to the nanoscale	Bulk powders, Nanowire bundles	Biomedical	(Yang et al. 2019)

Figure 2: Nanoparticles based on dimensions (Pal et al., 2011)



Based on uniformity: NPs can be classified in the forms of their states such as distributed aerosols, nanoclusters, colloidal solution and suspension (Kumar et al., 2018). These behaviors are based on the notion of dispersed phase and nature of dispersion medium along with their chemical and electromagnetic properties. Effectively, slightly loaded particles and magnetic particles display aggregation unless some stabilizing agent, like polymers are added and covers their surface. Agglomerated NPs act as macromolecules and loses the specific characteristics of NPs. NPs like nanocubes and spherical NPs are isometric due to their equal sizes (Adamiano et al., 2018,

Saleh et al., 2020). There are some anisometric NPs like nano-stars, nanorods and nano-plates etc (Bansal et al., 2020).

Metal Oxides NPs: For the last several decades, metal oxides have gained a considerable interest for the maximum numbers of nanomaterials due to their different promising constituents and versatile nature. Metal oxides are most easily available, fast and effective materials for their application in water treatment without the involvement of undesirable by-products. It can also be applied for many other applications including biomedical sciences because of their nontoxic and anti-microbial behavior. They can provide the oxygenated sites for the surface complexation with foreign elements thus can be most suitable for water remediation technology. Moreover, metal oxides due to their high surface area are expected to be more suitable for water treatment under the water quality constraints. For this, the separation of adsorbent and post adsorption is necessary; therefore, the use of magnetic metal oxide NPs in the field of water treatment technology has more pronouncedly emerged, with these characteristics the metal oxide NPs are further classified as follows:

Monometallic NPs: As the name suggests monometallic NPs (MNPs) consist of single metal atoms, which alone determines the properties of these NPs. They can be prepared by many methods, out of which chemical method is the most common. From the past few decades, MNPs have attained greater interest owing to their enhanced physical and chemical properties (Pantidos et al., 2014). The most important examples of monometallic oxides are: Iron oxide (FeO, Fe_2O_3 and Fe_3O_4), titanium oxide (TiO_2), aluminium oxide (Al_2O_3), zirconium oxide (ZrO_2), manganese dioxide (MnO, MnO_2 and Mn_2O_3), copper oxide (CuO and Cu_2O) and zinc oxide (ZnO). These NPs have been widely used for several applications such as in electronic, dye adsorption (Zhang et al., 2016), catalysis (Gawande et al., 2016), optical (Maruthupandy et al., 2017), and as antimicrobial agents against a few microorganisms such as *Escherichia coli* (Ribeiro et al., 2018) and *Streptococcus mutans* (Ramar et al., 2015, Lima et al., 2020).

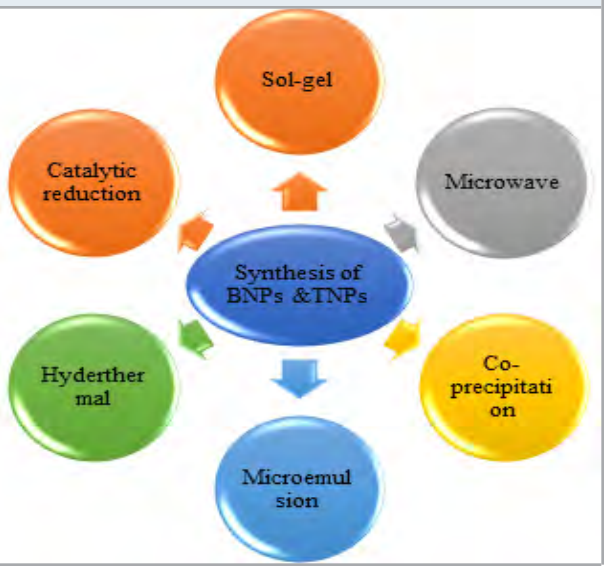
Bimetallic NPs and Trimetallic NPs: Bimetallic nanoparticle (BNPs) are the mixture of two different metals. BNPs can be formulated by using two inorganic materials in order to enhance the desired properties, which cannot be achieved by single metal atom. Furthermore, by the virtue of tiny size and greater volume to surface area ratio, these are significantly used in adsorption of various dyes for water purification, anticancer properties, catalyst etc. (Nasrabadi et al., 2016, Sharma et al., 2017).

In addition to magnetic adsorptive property, adsorbent used in removal of pollutants like arsenite As(III), preoxidation of As(III) to As(V) is also necessary, which can be achieved by the doping of oxidants like chlorine. However, they can also increase the risk of the formation of un-healthy by-products by reacts with natural organic matter present in water. Trimetallic NPs (TNPs) are the blend of three different metals and have advantage over MNPs. The volume to surface area ratio of TNPs is reasonably unstable, which can be stabilized by using different stabilizers such as organic ligands and surfactants leads (Martinez et al., 2018). TNPs as well as BNPs have acquired more interest than the MNPs in terms of scientific and technological point of view (Ravi et al., 2019). The properties of BNPs and TNPs can be same or differ from the pure elemental particles and may acquire unique size, and extra optical, electronic, thermal and catalytic properties (Sharma et al., 2017, Ali et al., 2020).

In past few years, extensive studies in the field of BNPs and TNPs have recorded.

Preparation of Bimetallic and Trimetallic NPs: BNPs and TNPs can be synthesized by various important methods such as sol-gel, microwave radiation, co-precipitation, catalytic reduction and hydrothermal etc. These methods are important to prepare nanoparticles of different size, shape and composition. Some of the methods used for the synthesis of the nanoparticles are discussed below (Table 1.2 and Fig.3).

Figure 3: Different approaches of synthesis for Bimetallic and Trimetallic NPs.



Applications of Bimetallic and Trimetallic nanoparticles: NPs because of their versatile properties and many folds enhanced chemical, catalytic, structural, magnetic and electrical characteristics, they find wide applications in the field of electronic, optical, energy, biological, medicinal and environmental industries (Fig.4). Among these applications, our review discusses the use of these BNPs and TNPs in the field of environmental pollution (water treatment) and nanomedicine for the treatment of lethal diseases, (Chen et al. 2016).

Application of BNPs and TNPs in Water treatment: Industrial wastewater is the major source of water pollution. As per the WHO report (WHO), thousands of

pollutants are present in environment in the form of air and water pollutants, some of these pollutants have severe thread for living organisms specially for aquatic systems, besides these pollutants are also responsible for various types of deadly diseases like cancer. Globally 9.6 million deaths per annum due to cancer in 2018 (WHO 2018). The alarming death rate due to cancer has become a worldwide challenge to the bio scientists and physicians. Literature reports that the toxic dyes used in textile industries is one of the main sources of pollutants causing cancer and danger for aquatic life (Mishra et al. 2015).

Table 1.2. Fabrication, mode of synthesis and significant applications of some important inorganic and organic nanoparticles.

Sr. No.	Inorganic/ organic NPs	Mode of synthesis	Significant applications	Ref.
1	Titanium dioxide	Sol gel, Hydrothermal, sonochemical, solvothermal, reverse micelles	Photo-catalysis, antimicrobial applications, gas and humid sensor, sunscreen products, wastewater treatment etc.	(Morshed et al., 2018, Baranowska-Wójcik et al., 2020)
2	Zinc oxide	Homogeneous precipitation, microwave method, thermal evaporation, Sol–gel, and chemical synthesis	Gas and humid sensor, photocatalytic degradation of toxic pollutants from wastewater, skin care products, biomedical applications such as anticancer, antifungal and antibacterial etc.	(Rajabi et al., 2017)
3	Aluminium oxide	Sol–gel, flame spray pyrolysis, reverse micro emulsion	Removal of heavy metal ions from waste watertreatment, antimicrobial applications, separation chamber, catalysis, biomedical applications etc.	(Su et al. 2018)
4	Silica	Flame synthesis, sol-gel, micro emulsion	Gene and drug delivery, biosensor, enzyme immobilization etc.	(Sodipo et al.2016)
5	Magnetic	Sol–gel, Co-precipitation, solvothermal, and sonochemical method	Bio-separation, dye and arsenic removal from wastewater, MRI, Immobilization of enzymes	(Srikar et al.2016)
6	Silver	Chemical reduction, photochemical method, Microwave and gamma irradiation, Biological synthesis by plant extract, enzymes, carbohydrate etc.	Antimicrobial, anticancer, catalysis, biosensor, water purification	(Natsuki et al.2015)
7	Gold	Reduction by chemicals, photochemical reaction, microwave irradiation	Biosensors, catalysis, drug delivery, anticancer etc.	(De souza et al. 2019, Priyadarshini et al. 2017)

Continue Table 1

8	Starch	Acid Hydrolysis, Ultrasonication, Gamma Irradiation	Drug carrier, wastewater treatment, tire making, fat replacers and emulsion stabilizers	(Kim et al. 2016)
9	Chitosan	Ionic gelation method, Reverse micelles method	Delivery system for vaccines, prevent infection in wounds and wound-healing process by enhancing the growth of skin cells, antibacterial agent	(Chandra et al. 2016)

The effects of textile dyes on the wealth of society and aquatic systems have been shown in Fig. 5 (Sha et al. 2016). Thus, the water pollution acts as the biggest challenge to the mankind (Carolin et al. 2017).

Adsorption technique is found to be more suitable, simple and cost effective, which has been efficiently used for the removal of wide range of water pollutants (Lata et al. 2016).

Figure 4: Various applications of metallic NPs.

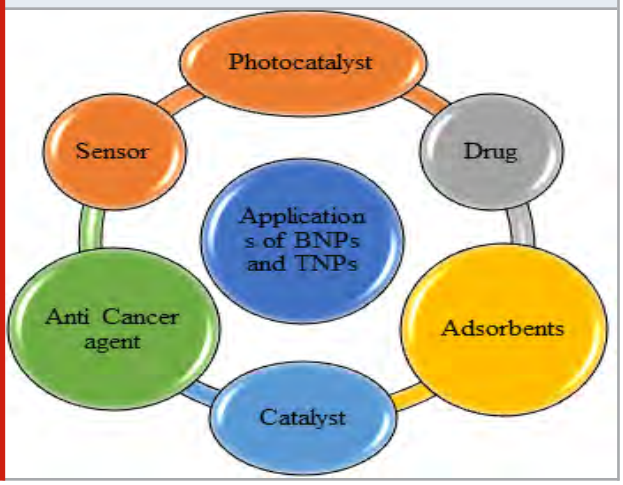


Figure 5: Pictorial representation of the effect of textile dyes in health and aquatic system.

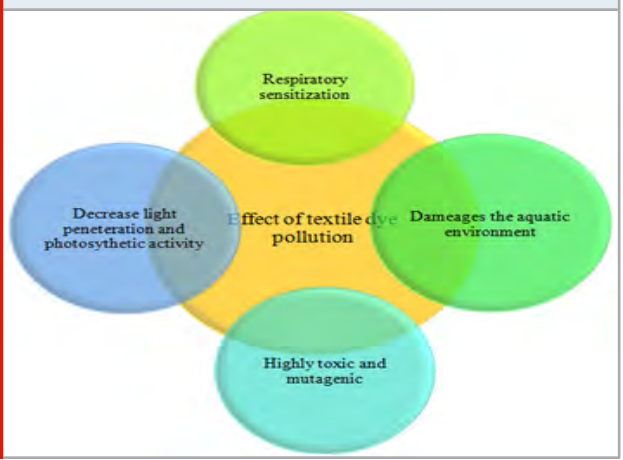


Table 1.3. List of adsorbents used against the removal of various dyes

S.N.	Adsorbent	Dyes	Ref.
1	Fe–Ni bimetallic NPs	Reactive Blue 21	(Kale et al. 2019)
2	ZnO NPs	Methyl orange Amaranth	(Zafar et al. 2019)
3	Partially oxidized graphite nanoparticles (POG-NPs)	Congo red Malachite green	(Mahmoud et al. 2019)
4	Fe_3O_4@SiO_2@PIL nanocomposite	Acid orange II Thionin acetate	(Yang et al. 2019)
5	ZnO	Malachite Green Congo Red	(Zhang et al. 2019)
6	$FexCo_3$-xO4 NPs	Congo Red	(Liu et al. 2019)
7	Ni-Ag bimetallic NPs		
	Sunset Yellow	(Mirzajani et al. 2019) Tartrazine	
8	Agar@Fe/Pd Bimetallic NPs	Methylene blue Rhodamine B	(Patra et al. 2019)

9	ZnO NPs stabilized on MWCNTs	Reactive blue 203	(Bagheri et al. 2019)
10	Ag-NPs using Albizia procera leaf extract	Methylene blue	(Rafique et al. 2019)
11	Fe_3O_4@Tb/AMP core-shell NPs	Alizarin Red Congo Red	(Huang et al. 2018)
12	CeO2 NPs	Reactive Green 19 Reactive Orange 84 Reactive violet 1 Reactive Yellow 81	(Sane et al. 2018)
13	WOx NPs	Rhodamine B	(Ying et al. 2018)
14	Alginate/γ-Fe_2O_3	Methylene Blue	(Talbot et al. 2018)
15	ZnO NPs using alginate	Methylene Blue	(Tamer et al. 2018)
16	Iron oxide NPs	Reactive Black 5	(Chang et al. 2018)
17	Nickel ferrite	Methyl orange Congo red	(Moghaddam et al. 2018)
18	carbon dots/$ZnFe_2O_4$ (CDs/ZFO)	Methyl orange	(Shi et al. 2018)
19	Zr-based magnetic Metal-Organic Frameworks composites	Methylene Blue	(Huang et al. 2018)
20	$SrFe_2O_4$	Erichrome black T Methylene blue	(Zafar et al. 2018)
21	ZnO	Congo red Brilliant green	(Kataria et al. 2017)
22	ZnO loaded activated carbon	Orange G Rhodamine B	(Nasrollahzadeh et al. 2018)
23	$NiFe_2O_4$@AlMCM-41-Cu_2O	Methylene blue	(Sohrabnezhad et al. 2017)
24	$MnFe_2O_4$/diatomite nanocomposite	Methylene blue	(Sun et al. 2017)
25	Ag NPs	Methylene blue	(Saha et al. 2017)

These adsorbents maybe of organic (Cellulose, organic fibers, agricultural wastes and their fibers etc.) and inorganic (sands, metallic ferrites, metal sulphides, oxides etc.) nature (Soltani et al. 2015). Among these various types of magnetic metal ferrites have been successfully used for the removal of toxic dyes and other pollutants due to their easy reusability high absorbance capacity and more simple mode of application and rechargeability (Chang et al.,2020). The use of various types of metal NPs as adsorbents for the removal of different types of dyes are highlighted in Table 1.3.

Moreover, the literature on the application of various types of NPs in removal of dyes is highlighted in Table 1.3.

Future Applications: Bimetallic and trimetallic are very promising nanomaterials for waste water treatment as compared to other types of NPs. To get the best result further experiments and research should be carried out. Further, these NPs could be used for other applications including biomedical application and catalysis.

CONCLUSION

Bimetallic and Trimetallic nanoparticles are more important than that of the monometallic nanoaprticles. The bimetallic and trimetallic nanoparticles are synthesized by various method such as sol-gel, microemulsion, sputtering, co-precipitation etc. Different shape and size of the BNPs and TNPs can be obtained by the various methods. Bimetallic and Trimetallic NPs are used as an excellent adsorbent due to their high adsorbing capacity and shown outstanding results for reusability purpose.

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Fusarium Wilts Controlling Revealed Physiological and Biochemical Variations in Tomato (*Lycopersicon esculentum* L.) Cultivar

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ABSTRACT

The objective of the present investigation was to evaluate the effect of new fungicides on wilt incidence and yield of tomato. The experiment was carried out following standard randomized complete block design at the rate of three replications with eighteen new fungicides on BARI Tomato 14 variety. New fungicides significantly enhanced plant height (Magvit 80 WP: 57.44 cm), number of branches (Wonderful 80 WP: 11.67), number of fruit branches (Gunzim & Provax: 10.33), number of fruits (Provax: 40.33) and weight of fruits (Provax: 1209.90 g). The lowest wilt incidence and highest plant survival were recorded in Provax which was statistically similar to Gunzim, Ranazim 50 WP, T. Bendazim, One Sighn, Larkzim 50 WP, Descozim, Biozim 50 WP, Rexizim 50 WP, Sarazim and Rajvit. The highest incidence and the lowest plant survival were recorded in untreated control which was not statistically similar to other fungicides. Provax treated plots gave the highest (39.31 t/ha) yield, which was statistically identical to Gunzim, Ranazim 50 WP, T. Zeb, T. Bendazim, One Sighn, Larkzim 50 WP, Descozim, Biozim 50 WP, Rexizim 50 WP, Sarazim and Rajvit and untreated control gave the lowest yield which was not statistically identical to other fungicides.

KEY WORDS: FUSARIUM WILT, TOMATO, MORPHOLOGICAL CHARACTERIZATION, FUNGICIDES.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop next to potato (De et al., 1996; Amini 2009). It originally came from tropical area from Mexico to Peru. Tomato has achieved tremendous popularity throughout the world over the last century. It is one of the most widely grown vegetables, which is grown mainly in the open-field for home use and local markets (Kamal et al., 2009; Hossain et al., 2014). It is important cash crop grown by both small scale farmers and commercial growers for fresh market and processing industry (Lemma et al., 1992). It is beneficial to human health being rich in minerals, vitamins, essential amino acids, sugars and dietary fibers (Miller et al., 1986 Misra et al., 2008). It is popular delicious vegetable in Bangladesh. Bangladesh has got tropical and sub-tropical climate suitable for cultivation of tomato. But tomatoes are parasitized by a number of pathogens, including *Fusarium oxysporum sp lycopersici* causal agent of fusarium wilt. Some fungal seed borne pathogens have ability to kill the seedling or plants and substantially reduce the productive capacity (Assefa et al., 2015; Mane et al., 2020).

ARTICLE INFORMATION

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Among them most important fungus reported are *Fusarium solani* and *F. oxysporum* (Mane et al., 2018). *Fusarium* wilt is one of the most devastating diseases of crops of Solanaceae family in Bangladesh. It is one of the important disease causing yield reductions in the field (Ferniah et al., 2014). *Fusarium* wilt, caused by the soil-born fungus *Fusarium oxysporum*, initially causes a yellowing and wilting of lower leaves on infected plants. *Symptoms* can be seen on a single branch, or on several branches on one side of the plant, or on all the lower branches. Since *Fusarium* wilt is a serious threat a strategic crop in Bangladesh, effective control measures are searched. Several disease management strategies are available e.g. cultural technique, biological control, resistant cultivars, crop rotation and chemical control (Kamal et al., 2009, Assefa et al., 2015).

However, all management strategies were unproved in all ways due to highest resistance ability of the fungus. Even though, resistant cultivars are the most effective measure of controlling *Fusarium* wilt, but new races of the pathogen appear to overcome resistance genes in currently grown cultivars (Sanogo et al., 2003). In order to prevent and control the wilting of seedling and adult plant and to protect the crop plants against pathogens, chemical control methods were in practice in Bangladesh. Therefore, in the present research investigation, new fungicides on BARI Tomato 14 variety were selected as an effective candidate because of their unknown facts and mechanism of action with respect to the crop immunity and productivity under field condition.

MATERIAL AND METHODS

Host plant and field area: Tomatoes plants are highly susceptible to fungal diseases were grown in the field of Regional Agricultural Research Station, BARI, Ishurdi, Pabna. BARI Tomato 14 variety was used as test plant which was brought from local market. The experimental land was well ploughed and properly leveled before bed preparation. Weeds and stubbles were removed from the field. Cow dung @ 10 t/ha, Urea @550 kg/ha, TSP @ 450 kg/ha and MP @ 250 kg/ha were applied (Sanogo et al., 2003).

Field experiment: The experiment was carried out following randomized complete block design with three replications. Total of eighteen new fungicides were selected for the experiment and study were designed according to the standard alignments. The new fungicides are depicted in table 1. The treatments were applied in pits of tomato plant ten days after crop plantation. Size of the plots was 2.0 m × 1.2 m and plant spacing was 60 cm × 50 cm. Fusariam inoculum was mixed with soil before two weeks of plantation of tomatoes for establishment of fungi. Intercultural operations were done as per needed and to maintain the normal hygienic condition of crop in the field. Wilt incidence, number of healthy plant, plant height, number of branches/plant, number of fruit branches/plant, number of fruits/plant, weight of fruits/plant yield (t/ha) were recorded (Goldberg 2010).

Statistical analysis: The disease of tomato field percentage of infected plants and percentage of damaged plants was recorded by adopting the grading formula. The percentage of infected and damage plants were calculated by the formula:

$$\text{Disease incidence (\%)} = \frac{\text{Total No. of infected plants}}{\text{Total No. of plants}} \times 100$$

The recorded data were analyzed statistically to find out the level of significance and the variations among the respective data were compared following Duncan's New Multiple Range Test (DMRT) according to standard data.

RESULTS AND DISCUSSION

In the present investigation, the effects of eighteen new fungicides on BARI Tomato 14 variety were evaluated at Regional Agricultural Research Station, BARI, Ishurdi, Pabna. The effect showed by the new fungicides which were resulted into the enhanced growth of the tomato plants, in which fungicides promoted different traits in tomato plants such as number of branches, number of fruit branches, number of fruits and weight of fruits. It happened due to more efficient genes available in the tomato plants and triggering by new fungicides, ultimately plants depicted more competitive in suppressing wilt incidences caused by the soil-born fungus *Fusarium oxysporum*. The *Fusarium* wilt caused by *F. oxysporum* sp. lycopersici is one of the serious diseases of tomato responsible for serious economic losses (Mane et al., 2018). The list of eighteen new fungicides is presented in table 1.

Table 1. List of new fungicides used on wilt incidence and yield of tomato

Treatments	Fungicides	Efficient (g/L)	Concentrations
1	Gunzim	Carbendazim	2
2	Wellvit 80 WP	Sulphar	2
3	Wonderful 80 WP	Sulphar	2
4	Ranazim 50 WP	Carbendazim	2
5	Zafer 80 DF	Sulphar	2
6	T. Zeb	Sulphar	2
7	T. Sulphar	Sulphar	2
8	T. Bendazim	Carbendazim	2
9	Zeesul 80 WG	Sulphar	2
10	One Sighn	Carbendazim	2
11	Larkzim 50 WP	Carbendazim	2
12	Magvit 80 WP	Sulphar	2
13	Descozim	Carbendazim	2
14	Biozim 50 WP	Carbendazim	2
15	Rexizim 50 WP	Carbendazim	2
16	Sarazim	Carbendazim	2
17	Rajvit	Sulphar	2
18	Provax 200	- 2	

The lowest wilt incidence (4.64%) and highest plant survival (95.76%) were recorded in Provax which was statistically similar to Gunzim, Ranazim 50 WP, T. Bendazim, One Sighn, Larkzim 50 WP, Descozim, Biozim 50 WP, Rexizim 50 WP, Sarazim and Rajvit and the highest incidence was (40.90%) and the lowest plant survival (59.10%) was recorded in untreated soil as control which was not statistically similar to other fungicides. The results are depicted in table 2. The variability among five isolates of *Fusarium solani* causing root rot of mulberry and found that five *Fusarium solani* isolates resulted 40.00-55.00 mm radial mycelial growth at 7 days after inoculation (Ferniah et al., 2014), while others measured 30 mm radial growth of *Fusarium oxysporum* f. *sp. phaseoli* after 72 hrs of inoculation (Yadeta et al., 2013). It was also recorded that fungal colonization by the inducer microorganism was necessary before resistance could be realized (Mane et al., 2020).

The importance of a competence depth of the inducer was up to 8 cm in relation to infection of *Fusarium oxysporum* (Goldberg NP. 2010). Immunity of the tomato plants was enhanced due to new fungicides and vigour compared to the untreated control. The *Fusarium oxysporum*, *F. pallidoroseum* and *Rhizoctonia solani* were consistently associated with higher frequency with the diseased parts (Soboka et al., 2012).

The field experiment with three fungicides at 0.5% concentration in randomized block design where application of Bavistin showed up to 62.27% reduction of wilt infection in tomato plants (Ferniah et al., 2014; Abada et al., 2014). Meanwhile other projects revealed that coating of chickpea seeds with Carbendazim (0.2%) was more effective in reducing wilt and increasing seed yield by 25.9 to 42.6 percent (Hossain et al., 2014). But the management of wilt incidences and deficient plant traits is a challenge as it behaves differently from other fungi and is a soil borne-systemically infecting pathogen. Considering use of new fungicides in this study as innovative approach to manage phytopathogens, in order to manage fungal diseases, it is important to screen antifungal activity of such fungicides to confirm the effectiveness (Kamal et al., 2009).

Table 2. Effect of fungicides on wilt incidences and yield contributing characters of tomato plant

Treatments	Wilted plants (%)	No. of fruit branches/ plant	No. of fruits/ plant	Wt. of fruits/ plant (g)
Gunzim	5.87 g*	10.33	36.81 cde	1004.30 bcd
Wellvit 80 WP	16.03 bcd	9.00 ab	32.20 h	966.12 cd
Wonderful 80 WP	12.80 de	8.33 abc	33.00 gh	990.00 bcd
Ranazim 50 WP	6.43 g	7.67 bc	37.48 b-e	1134.30 ab
Zafer 80 DF	16.91 bc	8.00 bc	32.00 h	960.23 cd
T. Zeb	10.35 ef	7.67 bc	36.13 cde	1083.90 abc
T. Sulphar	16.69 bc	7.00 bc	32.93 gh	948.12 cd
T. Bendazim	6.70 g	7.00 bc	36.20 cde	1089.00 abc
Zeesul 80 WG	18.58 b	8.67 abc	33.67 fgh	990.00 bcd
One Sighn	5.73 g	8.00 bc	37.47 b-e	1104.00 abc
Larkzim 50 WP	4.89 g	7.00 bc	39.34 ab	1190.10 a
Magvit 80 WP	13.41 cde	8.00 bc	35.12 efg	1003.50 bcd
Descozim	7.32 fg	7.67 bc	35.85 def	1075.50 abc
Biozim 50 WP	5.55 g	7.67 bc	36.91 cde	1107.30 abc
Rexizim 50 WP	5.24 g	8.33 abc	37.90 bcd	1137.00 ab
Sarazim	6.91 g	8.00 bc	38.34 abc	1168.20 a
Rajvit	5.77 g	7.67 bc	39.33 ab	1176.67 a
Provax 200	4.64 g	10.33 a	40.33 a	1209.90 a
Control	40.90 a	6.67 c	27.78 i	850.63 d
CV (%)	13.33	14.43	2.67	7.65
LSD (P≥0.05)	3.284	1.928	2.118	134.5

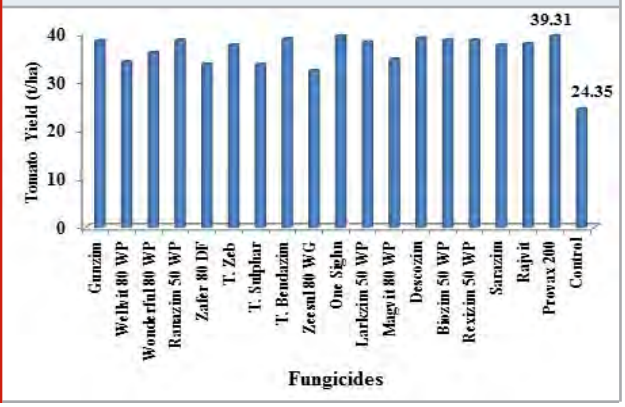
*In a column, similar letters do not differ significantly at 5% level of probability

Tomato plant height was significantly influenced by the fungicides but not number of branches per plant. Magvit 80 WP treated plots gave the tallest plant (57.44 cm) which was followed by maximum fungicides treated plots and control treatment gave the smallest plants (48.22 cm). The maximum (11.67) number of branches per plant was obtained from Wonderful 80 WP and T. Bendazim and the minimum number (9.33) of branches

per plant was obtained from Ranazim 50 WP and T. Zeb. The results are depicted in Table 2.

The highest (10.33) number of fruit branches per plant was recorded in Gunzim and Provax which was followed by Wellvit 80 WP, Wonderful 80 WP, Zeesul 80 WG and Rexizim 50 WP, and the lowest (6.67) number of fruit branches per plant was recorded in untreated control. Provax 200 resulted the highest (40.33) number of fruits per plant which was statistically identical to Larkzim 50 WP, Sarazim and Rajvit, and untreated control resulted the lowest (27.78) number of fruits per plant which also was not statistically identical to other fungicides. The highest (1209.90 g) weight of fruits per plant was obtained from Provax treated plots which was followed by Ranazim 50 WP, T. Zeb, T. Bendazim, One Sighn, Larkzim 50 WP, Descozim, Biozim 50 WP, Rexizim 50 WP and Sarazim, and the lowest (850.63 g) weight of fruits per plant was obtained from control treatment. The results are depicted in Table 2. Provax treated plots gave the highest (39.31 t/ha) yield which was statistically identical to Gunzim, Ranazim 50 WP, T. Zeb, T. Bendazim, One Sighn, Larkzim 50 WP, Descozim, Biozim 50 WP, Rexizim 50 WP, Sarazim and Rajvit and untreated control gave the lowest (24.35 t/ha) yield which was not statistically identical to other fungicides. The results are depicted in Fig. 1.

Figure 1: Effect of Fungicides on yield of tomato



Tomato plant growth mainly depends upon storage substance like carbohydrates, which are mobilized in the outline of soluble sugars (Lemma et al., 1992). Tomato plant verities demonstrated variations in the composition of soluble sugar when subjected to different spells of pathogenic conditions (Mane et al., 2020). The drop in glucose, fructose and sucrose concentration and an increased accumulation of sorbitol level accounts a common response of tomato plants in response to pathogenic conditions (Mane et al., 2018).

The newly used fungicides played a very important role in the immunity system of tomato plants by increasing their carbohydrate level and other protein levels. It happened due to carbohydrate equilibrium showed by tomato plants after treatment therefore they showed effective growth of branches, fruit branches, fruits and weight of fruits. Others evaluated some fungicides thiram, emissan (2 methoxy ethyl mercury chloride), indofil M-45 80% WP (mancozeb + thiophanate-methyl), captaf 50% WP (captan) and bavistin 50% WP, alone or in combination for their effects on tomato foot rot in vitro (Sanogo et al., 2003, Mane et al., 2020). The present study highlights the efficiency of the tested new fungicides in inducing resistance against fungus *Fusarium oxysporum* in tomato plants after field trials. Although the complete mechanism of action of new fungicides is yet to be elucidated, the results confirm a new biological action of the new fungicides. The present investigation will help in understanding the pathogenesis and molecular basis of defense reactions in tomato plants against fungal infections and in the selection of traits in plant breeding programmes.

CONCLUSION

The present investigation revealed the efficiency of the Provax which gave the highest (39.31 t/ha) with lowest wilt incidence and highest plant survival. Gunzim, Ranazim 50 WP, T. Bendazim, One Sighn, Larkzim 50 WP, Descozim, Biozim 50 WP, Rexizim 50 WP, Sarazim and Rajvit are also performed better for controlling Fusarium wilt of tomato. Moreover, the activity of the fungicides was effective and successfully controlled the fusarium wilt of tomato. Overall, our findings have provided the basis for controlling the fusarium wilt of tomato to untangle the decreased yield of tomato.

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Wildlife Hunting by Indigenous Tribes: a Case Analysis from Susunia Hills, West Bengal, India

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ABSTRACT

Hunting of wild animals from wilderness for sustenance is the common practice of many tribal communities. Indigenous tribal communities were engaged in hunting for many reasons like food, medicine, trade, culture and leisure. In the present communication an attempt has been made to document the traditional knowledge of tribal people which were inherited from generation to generation among the tribal residing in the villages surrounding Susunia hills. The study was conducted during August, 2019 to December, 2019 covering five villages around Susunia hills of Bankura. The survey, group discussion and data collection method were followed by a standard set of questionnaires. A total of 56 tribal people responded to our queries on different aspects of hunting, information on hunting gears, hunting practices and species targeted for hunting. The information was collected by interviewing aged and knowledgeable tribal people. A total of 36 species of animals were documented which were used by them during wildlife hunting. Various fossil remains were also collected and documented by Zoological survey of India from the Indian Pleistocene alluvial deposits from the surrounding villages of Susunia hill. It has also been concluded from the study that due to improvement of socio-economic status, betterment of educational and other governmental facilities and easy availability of alternate sources of protein from domestic meat (poultry birds) has refrained new generations of tribal people from undergoing hunting practices. It is also proposed from the study that if the scope of ecotourism is developed in Susunia hills then tribal people surrounding hills may further be benefitted and it will indirectly help to protect the wild animals of Susunia hills.

KEY WORDS: ECOTOURISM, HABITAT, HUNTING, INDIGENOUS, SUSUNIA, TRIBAL.

INTRODUCTION

India has richest floral and faunal diversity but now the country's biodiversity is rapidly declining due to human interferences and habitat destruction. Various tribal communities collect food and natural resources from nature and in this process knowingly or unknowingly destroying wild animals in different parts of the world.

ARTICLE INFORMATION

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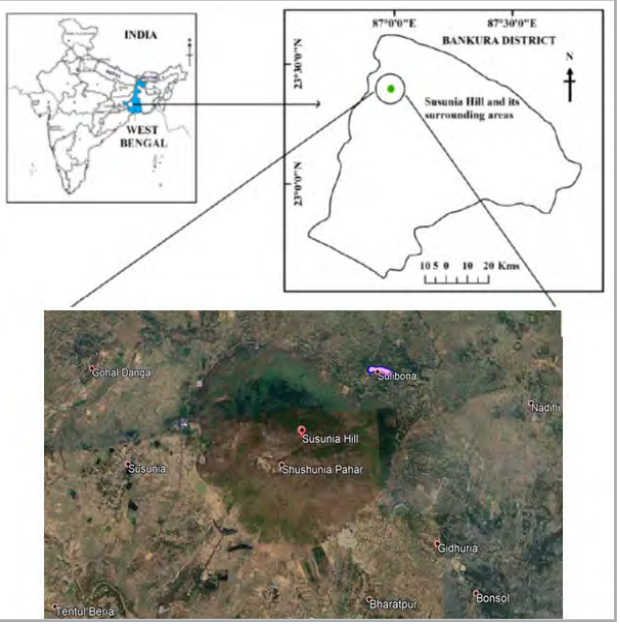
Different tribal groups capture or kill wild animals like mammals, birds, amphibian and reptiles by various hunting technique. They use these wild animals to obtain protein supplements in diet as meat, socio cultural celebrations, folk medicines, decoration purpose or just. Therefore, the excessive rates of utilization of the animal species affect the population of those species and the entire biological community of the region as well fun (Siddiq et al., 2018; D'Cruze et al., 2018; Loke et al., 2020).

In recent decades the sustenance of wildlife has been taken over by commercial utilization which is the most common cause of animals' extinction and habitat destruction (Baille and Aunger, 1989; Foster and Machilis, 1996; Harcourt and Park, 2003). From prehistoric time human hunted wild animals for their own need. In the beginning of mankind, the hunting was mainly done for food and

survival. But in recent decades it is mainly done for economic purpose or recreation (Robinson and Redford, 1994; Bodmer et al., 1997). Hunting of wild animals is common among tribal people in different countries like Vietnam, China, Indonesia, Yunnan, West and Central Africa and South America (Eudey,1999; Peres, 2000; Loke et al., 2020). This practice is also very common among various tribal groups residing in India (Borang, 1996; Harit, 2002; Solanki and Chutia, 2004; Singh et al., 2018). Wildlife hunting practice is also observed in different districts in the state of West Bengal.

But not much information is available about the wildlife hunting practices among the tribes residing in the vicinity of Susunia hills. So, the present study has been conducted to document the knowledge base, hunting practices, indigenous hunting tools and techniques of tribes used generation after generation residing surrounding Susunia hills. The present study also tries to document the diversity of wild animals found surrounding Susunia hills of Bankura District, West Bengal. Such type of local knowledge and practices need to be analyzed minutely to develop a better wildlife management practice to conserve biodiversity in any place (Supple and Shapiro, 2018; Jugli et al., 2020).

Figure 1: Satellite image and map showing the study area



MATERIAL AND METHODS

The study has been carried out in three tribal villages, surrounding the Susania hill of Bankura district. Susunia hill (23.39693° N and 86.98527° E) is situated in the north-west of Bankura district, in the Chota Nagpur Plateau of West Bengal and rises to 439.5 m above sea level (Fig.-1). It is a moderate sized hill and runs for a length of about 3 km. Like other forest areas in the district, forest of the Susunia hill is also tropical dry deciduous type dominated by Sal tree (*Shorea robusta* Gaertn. f.).

The hill is very rich in its plant resources including medicinal plants. Bankura district is inhabited by many tribal communities such as Santhals, Oraons, Koras, Bhumij, Mech, Mahali, Bedia and Mundas. Santhals represent the largest indigenous tribal community in the district and the villages surrounding the Susunia hill are dominated by this tribe. The tribal villages selected for this study are located within 10 km radius around the hill (Jugli et al., 2020).

The study was conducted during August, 2019 to December, 2019 covering 5 villages surrounding Susunia hills of Bankura district namely Susunia, Seulibona, Bharatpur, Gidhuria and Gohaldanga in the State of West Bengal, India. Santhal tribes are found in the whole district, especially in southern part which is the area of our present study. The study area is mostly covered by forest with trees like Sal (*Shorea robusta*), Mahua (*Bassia latifolia*), Kend (*Diospyros melanoxylon*), Palas (*Butea frondosa*), Piasal (*Pterocarpus marsupium*), Simul (Bombax ceiba), Gamar (*Gmelina arborea*) and many medicinal plants (Mondal et al., 2016).

Table 1. Demography of the Respondents

Respondent's age	Family size (Average)	Earning member	Monthly income per member (Rs.)
35.5 (± 12.3) (Average)	5 ± 1.00	3 ± 1.00	5500 ± 500.00

Most tribal people earn their livelihood by working as daily labour engaged in collecting leaves of Sal, Kendu and various medicinal plant parts from the surrounding Susunia hills. During monsoon, many tribal people migrate to neighbouring district of Burdwan to work as daily labour. Occasionally, they do wildlife hunting in Susunia hills for meat and also as a part of their traditional festivals. During survey work, information's were collected by group discussions with tribal villagers. During survey work tribal individuals with different age group were selected randomly. The data collection method includes questionnaire, interview schedule, observation, and discussion with the respondents and literature. Study is based on primary data (Mondal et al., 2016).

For the collec¬tion of relevant data, a detailed questionnaire was prepared involving various aspects such as hunting methods, hunting tools and preference of animal selection during hunting, hazards and socio-economic status of tribes. Only adults of varying age and sex were selected because some of the questions were too difficult to answer for younger people. Before interview all the questions were clearly discussed with the respondents to avoid error. The survey was conducted during their leisure time when they were not in stress and willing to take their time for answering all the

questions. Filling in the questionnaire took between 10 to 15 minutes time for each respondent.

RESULTS AND DISCUSSION

Fossil fauna recorded surrounding hills: Various fossil remains were collected from the Indian Pleistocene alluvial deposits from surrounding villages of Susunia hills, Bankura, West Bengal by the Palaeozoology Division of the Zoological Survey of India. Although most of the fossils are decomposed, but the identified fossils proved to be the presence of Giraffe camelopardalis Linn.), Bos namadicus Falconer, Bos indicus Linn., Panthera purdus (Linn.), Equus sp. and many other species in pre historic time (Saha et al., 1984; Banerjee et al., 1987; Mondal et al., 2016).

Demography of the Respondents: A total of 56 tribal responded to our queries on the different aspects of hunting. Male persons are exclusively involved in hunting. Age of the participants ranges from 16–57 years. The mean age of the hunter is 35.5 (± 12.3) years. The age group of hunters between 26 to 35 years is most common. Family size varies from 3 to 12 members. Both male and female members work as unskilled labour in the agricultural field during monsoon season (Table -1). Other profession observed among them are, basket making by bamboo, dry wood, leaf collection from nearby jungles and 100 days worker under MGNERGA Scheme (Mondal et al., 2016).

Hunting attributes or habits: From discussion with tribal people it is observed that they did not follow a specific hunting schedule but do hunting as per their convenience, although some hunting trips were carried out for cultural or ritualistic reasons such as during village festivals and functions. During these periods hunting trips occur more often. April and May month is the preferred hunting time for most hunters (85%). Early morning is the preferred time to hunt. But for catching rabbits and birds they select the time during sunset. Older age group tribal people travel more during hunting trips in comparison to younger new generation hunters. Earlier hunters often used to travel 15 km or more from their villages, whereas now-a-days new generation hunters mentioned that they rarely travel distances of 2-5 km from their villages during hunting trip (Jugli et al., 2020; Loke et al., 2020).

Wildlife hunting by indigenous tribes: All the tribal groups under the study area hunted various wild animals surrounding the natural habitat of Susunia hills. A total of 36 species (Table-2) of animals were documented to be killed during hunting. The number of animals would increase many times if we include fish, insect and Mollusca. Among the animals, the major groups extracted were mammals than other animals (Ghosh et al., 1996; Ghosh et al., 2013). Occasionally few reptiles and amphibian were killed by them for meat. The villagers collect a wide range of animals from the forest and consume their flesh for food. The remaining body parts (bone, skull, skin, and hoof) were used for aesthetic purposes.

People use several articles, such as bags, skull caps and other headgear, made from animal parts. In addition, many birds and reptiles were also reported to be hunted. They decorate their room interior with the horn and skin of various animals. The skulls of the hunted animals were hung in front of the house as a symbol of prestige and for aesthetic purpose (Fig. -2). They use the colorful feather of the birds to decorate their room. During hunting they usually prefer Indian wild rabbit *Lepus nigricollis* among wild animals for its tasty meat. Traditional hunting gear: During hunting trips the tools used by them were mostly prepared from natural things like bamboo stick and many other plant parts but now days they use nylon net for catching wild hare (Fig. -3), iron wire for rats and sticky gum collected from various plants for capturing water birds and other birds near water body. For catching fishes' various traps were used which were prepared by bamboo stick. For killing larger mammals, they usually used lancet with pointed tips (Fig. 4).

Figure 3: Nylon net for catching wild hare

Figure 2: Skull of animals hung in front of the house

Figure 4: Traditional hunting gear used by tribal peoples

Table 2. List of animals hunted by villagers surrounding Susunia hills

Sl. No.	Class	Local name (Bengali)	Common name (English)	Scientific name
1.	Mammals	Bonno Shukor	Wild boar	*Sus scrofa*
2.		Bon Beral	Wild Cat	*Felis chaus*
3.		Sheal (Jackel)	Jackel	*Canis aureus*
4.		Khakseyal(Fox)	Fox	*Vulpas benghalensis*
5.		Badur	Bat	*Pteropus giganteus*
6.		Kat Birali	Squirrel	*Funambulus palmarum*
7.		Indure	Rat	*Bandicota benghalensis*
				Bandicota indica
				Rattus
				Rattus norvegicus
8.		Shojaru	Porcupine	*Hystrix indica*
9.		Beeji	Small Indian mongoose	*Herpestes auropunctatus*
10.		Sarsara	Wild hare	*Lepus nigricollis*
11..		Gandho gokul	Palm civet	*Paradoxurus sp*
12.		Chamchike	Small bat	*Pipistrellus coromandra*
13.		kotas	Large Indian civet	*Viverra zibetha*
14.	Bird	Ghugu	Eurasian collared dove	*Streptopelia decaocto*
15.		Payra	Pigeon	*Columba livia*
16.		Charai	Sparrow	*Passer domesticus*
17.		Chitghugu	Spotted dove	*Streptopelia chinensis*
18.		Shalik	Pied myna	*Gracupica contra*
19.		Jal murgi	White-breasted waterhen	*Amaurornis phoenicurus*
20		Hariyal	Yellow-footed green pigeon	*Treron phoenicoptera*
21		Tethoi pakhi	Yellow-wattled lapwing	*Vanellus malabaricus*
22.		Bon morag	Red junglefowl	*Gallus*
23.		Chatare	Jungle babbler	*Turdoides striata*
24.		bok	Indian pond heron	*Ardeola grayii*
25.		Gobok	Cattle egret	*Bubulcus ibis*
26.		Sada bok	Little egret	*Egretta garzetta*
27		Pankouri	Indian cormorant	*Phalacrocorax fuscicollis*
28.		Karkate pakhi	Brown shrike	*Lanius cristatus*
29		Kalo jalmurgi	Common moorhen	*Gallinula chlorotus*
30.	Reptile	Gosaap	Common Indian Monitor	*Varanu benghalensis*
31.		Ojogor	Reticulated python	*Python reticulatus*
32.		Jal dhora	grass snake or water snake	*Natrix sp.*
33.		Tiktiki	Common house gecko	*Hemidactylus frenatus*
34.		Girgiti	Oriental garden lizard	*Calotes varsicolar*
35.	Amphibia	Shona Bang	Indian bullfrog	*Rana tigrina*
36		Kuno Bang	Asian common toad	*Duttaphrynus melanostictus*

Hunting technique: The distance between village and hunting sites in forest is one of the major factors that influence the frequency of hunting. The distances of villages from their hunting arena are not far. During hunting they make a group of 5 to 10 member, often may be a large group containing 20-30 members and invade forest area or nearby agricultural field and killed wild animals in a group. During their hunting a beautiful co-ordination was observed and they communicate with each other by using some symbolic sound. Usually during full moon, they invade jungle in a small group and use various traps for catching wild animals. After completion of hunting they cut the animals in to pieces and distribute the meat equally among each other.

Present status of hunting: From our study we have observed that frequency of hunting trips has slightly decreases now a days. The reasons behind these are firstly, number of human populations have increased in each village. So, dependency on wild animal's flesh as food has reduced. Secondly if many members participated in hunting then share of flesh, they get is not sufficient for the whole family. New generation hunter is not interested with this type of uncertain hard

work and as new job opportunities like 100-day work under MGNERGA Scheme and unskilled labour work opportunities have increased. So, their socioeconomic status has slightly improved. In addition, availability of poultry birds has also increased in villages. So, they can easily collect poultry meat or digestive part and skin of poultry birds by spending little amount of money. Hence the uncertainty of hunting practices to obtain animal flesh has decreased. Lastly decrease in number of wildlife species, strong implementation of wildlife protection laws, increase of educational level and awareness among new generation tribal people has refrained them from hunting practices in these regions.

CONCLUSION

This study provides new information on hunting practices, species harvested and the socio-economic factors affecting the exploitation of wildlife surrounding the Susunia hills, Bankura. It also highlights the cultural use of wildlife by the district's indigenous groups. This could prevent local extinction of some species, especially in remote regions where law enforcement is weak and long-term hunting has already reduced wildlife populations. Thus, despite some of the limitations of our study, the information obtained is novel and may be valuable for future studies in this region. There was little wariness amongst respondents in answering questions about hunting. In few villages people were not prepared to discuss their hunting practices with us. The level of awareness about hunting laws is generally lower among older people but in new generation it is moderate. The easy availability of alternate sources of protein from domestic meat (poultry birds) has now decreased the hunting practices. Susunia hill is an ideal ecotourism destination where local people could be employed. Exploring the natural beauties of this hill to the world not only will bring a new area for West Bengal tourism but also tribal people of surrounding the hills may see the new light for socio- economic development having behind the illegal hunting activity.

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The Awareness Level of Saudi Housewives Towards Environmental Sustainability: The Relevance with their Practices to Maintain the Environment

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ABSTRACT

Environmental sustainability is one of the main strategic goals the Saudi Arabia is trying to meet, incorporated in the national transformation program to achieve the Kingdom's vision by 2030. For ages, humans have mistreated and damaged the environment, and thus for many acute ecological problems have surfaced. Hence the research was inspired. The purpose of this research is to find out the relation between the Saudi housewives' sustainability level of awareness and the actual practices they perform to preserve the environment, also to identify the differences between the impact of demographic variables on both environmental awareness and the procedures carried out by the Saudi housewives. Two questionnaires were filled by 200 Saudi housewives - Jeddah residence. The findings showed statistical significances from 0.001 to 0.05 between the environmental sustainability awareness level in the elements of (air, food, noise pollution & the exploitations of natural resources) and the actual practices performed by the housewives to preserve the environment and its resources, taking into account their career life, educational, social & economic backgrounds. According to the findings, the sustainability awareness level should be risen, incorporating it in the women & family programs using all kinds of media & social media platforms, educational curriculums, and finally put a strategy to create an integrated system to preserve the environment.

KEY WORDS: POLLUTION, ENVIRONMENT, PRACTICES, SUSTAINABILITY, HOUSEWIVES, AWARENESS.

INTRODUCTION

Environmental sustainability is one of the strategic goals the Saudi Kingdom is trying to incorporated in the national transformation program to achieve the Kingdom's vision by 2030. Caring for the environment and protecting it from all kinds of pollution is one of the most important and crucial national and international causes that have plagued the scientists for centuries. However, humans have mistreated the environment and performed many unfair practices, to meet their needs and lead a luxurious life, which has led to natural resources depletion, resulting in wastes and pollutants which deflected back on the environment (Rebecca, 2020). Those wastes and pollutants caused the environmental pollution problem. Thus, environmental sustainability and keeping it from all kinds of pollution, waste, and misuse of its resources, is one of the most pressing causes in modern history and a fundamental challenge facing the upcoming generations (Nathanson, 2000 and Marma, 2019).

The most problematic environmental issues are air, food, water & noise pollution, besides the depletion and waste of resources, as the human activities are draining the

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natural resources; it didn't stop there. It went to the extent of destroying nature, which is threatening the existence of humankind itself in the long run if we didn't comply with the environmental sustainability methods (Scientific and cultural organization, 2018).

As noticed that most of these problems are caused by the short-term human strategies focusing only on increasing production levels, without taking into account the adverse outcomes of these practices on the environment (Holum, 2017). The environmental effect on human life is bound by his sustainability awareness level, as he is the number one factor in the ecological status and the first to be affected by it as well (Kingdom of Saudi Arabia UN High-Level Political, 2018). Thus, it's evident the importance of the environmental sustainability awareness to face pollution problems and reach sustainability, as this is the only solution to preserve the life of the current organisms and the upcoming generations as well (Khalil, 2019).

Sustainability awareness is the level of understanding the possesses of the natural surroundings human. It can be defined as the interaction between the humans with the environment without the depletion of its natural resources and preserving the planet for as long as we can (Middle East Institute Viewpoint, 2011). Daud (2017) stated that the idea of humankind's existence on this planet is very much bound by the perseverance of the natural resources. There is importance for sustainability awareness in the national or international communities, the more immense responsibility lies on all family members and particularly on the housewife's, as she is the one responsible for the family's consumption habits dynamics. Also, the vital role she play in raising her children with the right morals, motives, behavioral dynamics, and the sustainable or unsustainable use of natural resources (John, 2017).

The importance of these practices with nature lies in the impact affecting life on Earth such as: Climate change (Blackwell and Chris, 2016). It's noteworthy that climate change affects every region differently, and all organisms (US Environment Protection Agency, 2017). Biodiversity extreme reduction level, habitat destruction, and pollution is currently ranked as the primary cause of species extinction worldwide and the incredible decrease in numbers for some others (Brito and Pinon 2012), as a result of the large-scale usage of pesticides that specifically affected bees and butterflies. Unsustainable consumption and production patterns, natural resources are being used globally with productions and consumptions patterns that involve converting the raw materials into commodities that are widely misused and consumed, then disposing of it recklessly, resulting in more pollutants and waste accumulations (Khan and Mujahid, 2011 and Islam et al., 2016).

Environmental elements pollution: It is one of the most severe environmental problems that arise from wrong practices, behaviors, and the fraudulent and unsafe usage of the environmental elements, such as: Air pollution: that leads to an undesired change in the natural properties of the air surrounding the domestic environment resulting from an increased percentage of pollutants such as dust, fumes. Cigarettes smoke, odors, commercial air fresheners, the use of toxins such as pesticides and others, which takes place by the family members themselves, especially the housewives (Stieb et al., 2002).

Food contamination affects the environment starting from the poor choices the housewife makes while purchasing food commodities, as checking the expiration date, the preservatives & artificial additives, or the purchase of exposed goods to environmental pollutants. This is in addition to not observing the hygienic & healthy regulations, which ultimately leads to a deterioration in the health of family members (Oskarsson, 2018). Noise pollution: which is one of the types of moral pollution, resulting from irrational usage of electrical devices such as using more than one electrical device at the same time, or turning the volume up of the television, radio, or cassette equipment, or use loudspeakers frequently (Chris et al., 2005).

Despite all efforts spent nationally by the Saudi Kingdom to preserve the environment from all kinds of pollution at all legislative, execution, and scientific levels, most studies refer to the need to change the group and family dynamics to face such problems or alleviate it, and to raise awareness of the relationship between humankind and the environment (Former, 2019). The research questions are determined in whether the level of awareness of the Saudi housewives of the concept of environmental sustainability has to do with their actual practices to preserve the environment from pollution if their behavior is affected by their social and economic status, what is the impact of the work, their educational background, and finally income, on the level of environmental awareness and the actual practices they take to preserve the environment from pollution?

The relation between the Saudi housewives' environmental sustainability level of awareness and the actual practices they perform to preserve the environment from different kinds of pollution (air pollution, food contamination, noise pollution, waste, and misuse of environmental resources) also introducing the correlation between demographic variables related to the (economic, educational, family income and the number of family members) and the practices of the housewives to protect the environment from pollution.

The research is a unique one in the field because it focused to the relation between housewives awareness of sustainability and their practices. It also sheds light on the most crucial factor that contributes to raising awareness of sustainability among the Saudi housewives and then placing them at the center of concerns in women and family programs through various media & social media platforms. This research assumes the existence of a statistically significant discrepancy in the averages of both awareness of environmental sustainability and the

practices of the working and non-working housewives to preserve the environment from pollution, and the existence of a positive correlation between demographic variables related to the economic, educational, family income and the number of family members, and the practices of the housewives.

It is also assumed that the relative measures of the variables of both awareness of sustainability and the practices of the housewives may differ. Preserving the environment from pollution according to the different types of those variables, and assuming the difference in the relative measures between each variable with its elements such as (air, food, noise, waste and depletion of natural resources) and the practices of the housewives to preserve the environment from pollution.

MATERIAL AND METHODS

This research followed a descriptive and analytical method.

Search limitations: Objective limits: a study of Saudi housewives' awareness of the concept of sustainability and the practices they perform to preserve the environment from pollution. Human limits: results are drawn from a self-administrated questionnaire of a random sample of 200 Saudi housewives. Spatial limits the research was applied in the Kingdom of Saudi Arabia- Jeddah residence. Time limits: This research was conducted from January 2020 and June 2020.

Research samples: The research examined two hundred (200) Saudi working & non-working housewives with different educational, socio-economic backgrounds and resided in various neighborhoods in Jeddah. They were randomly selected from the population of the research sample.

Table 1. Career life significance variables

Significance level	Significance type	Significance variable	Category
0.000	Significant	0.986	First
0.000	Significant	0.966	Second
0.000	Significant	0.978	Third
0.000	Significant	0.978	Fourth

Research tools: The data is collected through a self-administrated questionnaire including three parts : First part: General family information (education level, social level, economic level, career, and family income). Second part: Assessed the awareness level of sustainability included thirty-five (35) phrases distributed under four main categories: Air pollution awareness consists of 10 phrases; food contamination awareness consists of 9 phrases; noise pollution awareness consists of 7 phrases, and natural elements depletion and overconsumption awareness consist of 9 phrases.

This questionnaire measures the awareness level of the housewives of sustainability inside and outside their households. The responses were (agree, neutral, disagree) and on a related scale (1-2-3). The questionnaire was characterized by global & content validity, as the correlation coefficients of each category of the questionnaire and the total score of the questionnaire were statistically significant at the level of significance (0.01) Table (1). It is evident from Table (1) that all fields of the questionnaire, based on the level of significance, are considered transparent to what they have been measured. The questionnaire received a high value in its correlation coefficient Table (2). This confirms the consistency of the questionnaire and the reliability of the used techniques to measure sustainability awareness. Thus, supporting the validity of the data collected from the respondents

Third part: Assess the Saudi housewives' practices to protect the environment from pollution, the practices they perform to preserve the environment, and the alternatives they have within and outside the household, including thirty-two (32) phrases distributed under four main categories. Air pollution consists of 9 phrases; food contamination consists of 9 phrases; noise pollution consists of 6 phrases, and the natural elements depletion and overconsumption consists of (8) phrases. This questionnaire measures the practices housewives perform to preserve the internal & external environment of their households. The responses were (always - sometimes - rarely) and on a related scale (1-2-3).

The questionnaire was characterized by global & content validity, as the correlation coefficients of each category of the questionnaire and the total score of the questionnaire were statistically significant at the level of significance (0.01) Table (3).

Table 2. Cronbach's Alpha & split half to measure reliability

Category	Alpha Cronbach's Reliability Value		Half Split value	
	Number of phrases	Number of phrases	Persons Factor	Half Split Value
First	10	0.605	0.679	0.750
Second	9	0.819	0.708	0.828
Third	7	0.732	0.798	0.673
Fourth	9	0.655	0.586	0.550

Table 3. Career life significance variables

Category	Significance variable	Significance Type	Significance Level
First	0.875	Significant	0.000
Second	0.987	Significant	0.000
Third	0.690	Significant	0.000
Fourth	0.945	Significant	0.000

Figure 1: Distribution of housewives according to the educational level (A) and career (B) The first hypothesis

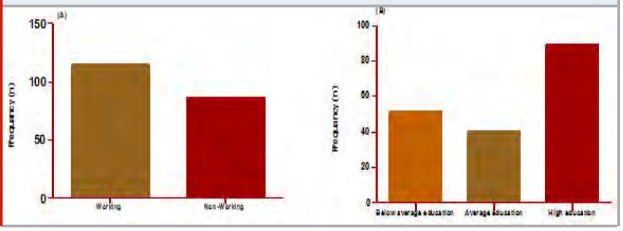

Table 4. Alpha Cronbach's & split half to measure reliability

Category	Alpha Cronbach's Reliability Value		Half Split value	
	Number of phrases	Alpha Cronbach's Value	Persons Factor	Half Split Value
First	9	0.563	0.760	0.871
Second	9	0.761	0.896	0.707
Third	8	0.568	0.970	0.870
Fourth	8	0.679	0.708	0.659

Table 5. Level of awareness of sustainability by the working & non-working housewives

Career	Mean	SD	Respondents	Freedom Level	(T) Value	Significance
Working	85.667	15.279	114	198	17.612	0.01
Non-working	50.883	11.619	86			

It is also evident from Table (4) that the questionnaire obtained a high value for the alpha coefficient and the half-segmentation It is evident from Table (4) to ensure the consistency of the questionnaire to measure the practices of the housewife's actual performed practices to preserve the environment. Implementation of the research tools on the respondents: The research tools were applied to the randomly selected working and non-working housewives, where some of them work at educational and administrative institutions. The study conducted during the months of the academic year (1440/1441 AH), taking into account the control and procedures following the instructions specified in the research tools

Table 6. The differences in the average of practices of the working and non-working housewives to preserve the environmen

Career	Mean	SD	Respondents	Freedom Level	(T) Value	Significance
Working	77.315	12.523	114	198	18.024	0.01
Non-working	47.151	10.551	86			

Statistical analysis: The statistical analysis program (SPSS Version 24) was used to analyze the study data, with the help of the necessary statistical methods, to verify the study hypotheses. These methods were as follows: Frequencies & percentages or mean± standard deviation to represented the data. Cronbach's alpha for the halftone segmentation method & split half. Pearson correlation coefficient to measure the validity of the internal consistency of the study items. One sample t- test: to check for statistically significant differences. T-test for two independent samples to check for statistically significant differences in the responses of the respondents due to the personal variables. One-way ANOVA test to check for statistically significant differences in the responses of the respondents due to the personal variables.

RESULTS AND DISCUSSION

Demographic data: It was found that the basic research included 200 Saudi housewives respondents split into (114 workings + 86 non-working), 51 housewives had below-average education with the percentage of 25.5%, another 60 housewives had received an average education level, with the percentage of 30%, and lastly 89 housewives who had high education degrees with the percentage of 44.5%, and they belong to families of different social and economic backgrounds Figure 1(A&B).

There are statistically significant differences in the averages of both awareness of the concept of sustainability and the practices performed by the working & non-working housewives''. The (T) value elaborates the significance of the differences between the averages of the awareness level of the working and non-working housewives of sustainability with its various elements (air, food and noise pollution, waste & depletion of the natural resources) and their practices to preserve the environment. The following Tables (5) and (6) illustrate this hypnosis: First: awareness of sustainability by the working & non-working housewives

Table (5) shows that there are statistically significant differences between the working and non-working housewives, where the value of t (17,612) was a significant level (0.01). That is, the level of awareness sustainability, in regards to environmental pollution problems inside and outside the household, it is higher among the working housewives compared to the non-working ones, and this may be due to the increased opportunities of social interaction among workers in the workplace and other areas compared to the non-working housewives, which raise their awareness, knowledge, and sense of environmental problems in terms of their causes, effects, and means. This result is in agreement with the study (Al-Zahhar, 2016), which indicated that there is a positive relationship between work and the enrichment of cognitive and emotional aspects.

The practices performed by the working and non-working Saudi housewives to preserve the environment from pollution: Table (6) shows that there are statistically significant differences between the practices of the Saudi working and non-working housewives in favor of the female workers, as the value of T (18.024) was significant at the level of significance (0.01), meaning that the practices performed by the housewives to preserve the environment are higher among the working ones compared to the non—working housewives. Some studies show statistically significant differences between working and non-working housewives in regards to making decisions related to consumer rationalization in the areas of food and resource conservation, and other show a positive effect the career life has on individuals, and from the previous review, it is evident that the first hypothesis has been partially fulfilled.

The second hypothesis ''the existence of a positive correlation between the demographic variables related to the socio-economic level (the educational level, income, and the number of the family members) and each of the variables of awareness of sustainability and the practices performed by the housewives to preserve the environment.''A matrix of correlation coefficients was found between the independent demographic variables related to the socio-economic level (educational level – age- the income - the number of the family members) and each of the variables of awareness of sustainability and the practices performed by the housewives to preserve the environment Table (7).

Table 7. Matrix correlation coefficients between the demographic variables and sustainability awareness of natural resources and the practices performed by the housewives to preserve the environment.

Demographic Variables	Sustainability awareness of natural resources					Practices performed to preserve the environment				
	Air	Food	Noise	Resources depletion	Whole	Air	Food	Noise	Resources depletion	Whole
Educational background	0.632*	0.823**	0.796**	0.906**	0.819**	0.915**	0.624*	0.845**	0.826**	0.882**
Age	0.867**	0.782**	0.643*	0.728**	0.772**	0.762**	0.793**	0.803**	0.619*	0.794**
Career Life	0.131	0.198	0.107	0.225	0.162	0.145	0.203	0.118	0.176	0.123
Family number	0.192	0.115	0.178	0.204	0.121	0.109	0.137	0.187	0.153	0.216
Income	**0.156**	0.213	0.182	0.105	0.168	0.718**	0.626*	0.774**	0.863**	0.725**

*** Significant at p<0.05. ** Significant at p< 0.001**

The existence of a positive correlation between the demographic variables related to the socio-economic level (the educational level of the housewives - the household income - the number of the family members) and each of the variables of awareness of sustainability and the practices performed by the housewives to preserve the environment. The educational backgrounds for these housewives, respectively (0.632), (0.823), (0.796), (0.906), (0.819), all of which are statistically significant at a level of significance between (0.05) & (0.001). This confirms that education contributes to raising the awareness level of sustainability. It is also evident from Table (7) that the value of (R) between the educational level of the housewives and the practices performed to preserve the

environment are: (0.915), (0.624), (0.845), (0.826), (0.882) respectively. All of them are statistically significant at a significance level between (0.05) & (0.001). That is, the higher the educational level of the family, the higher its ability to carry out practices that help preserve the environment. This indicates that the educational level of the housewives contributes to their wise and rational exploitation of the natural resources, and by referring to previous studies that dealt with the effect of education on environmental awareness and the formation of trends towards protecting the environment from pollution.

Afifi (2018) confirmed the results of this study, as they indicate the importance and power of education in forming sustainability awareness and making a positive change in the dynamics and behaviors of the individuals to protect the environment. As shown in Table (7), there is a positive correlation between the variable of family income and each of them performed practices to preserve the environment, as the value of (R) is (0.718), (0.626), (0.77), (0.863) respectively, and all of them are statistically significant at significance level between (0.05-0.001). Accordingly, the higher the level of the family's financial income, the higher the level of practices performed by the housewives to protect the environment from pollution, also having a positive behavior towards protecting the domestic environment. However, there is no correlation between these variables and the level of awareness of sustainability. It also shows that there is no correlation between the number of family members and the level of awareness sustainability, and the actual practices performed. Thus, the second hypnosis was partially fulfilled.

The third hypothesis,'the existence of a positive correlation between the variables of sustainability awareness and the practices performed by the housewives (research sample) to preserve the environment.'A matrix of correlation coefficients was found between the level of awareness of sustainability with all the elements (air, food, noise, environmental resources, and their depletion) and the actually performed practices to protect the domestic and external environment Table (8). Table (8) shows the existence of positive correlations between the variables of sustainability awareness and their ability to perform actual practices to preserve the domestic & external environment from pollution. The levels of significance between them and all the variables ranged between (0.01 and 0.05), which confirms the strength of the relationship between the housewives' awareness level of sustainability and the ability to perform actual practices to preserve the environment. Indicating that sustainability awareness, is positively related to the individual's attitudes towards protecting the environment, and it is also associated with the positive behavior represented in implementing rational and wise practices to protect the domestic and external environment.

Table 8. Matrix correlation coefficients for the awareness of environmental sustainability the actual performed practices to protect the domestic and external environment

Sustainability awareness level	Practices to Sustain the environment				Taking decisions to preserve the environment
	Air	Food	Noise	Natural resources depletion	
Air	0.737**	0.812**	0.904**	0.641*	0.702**
Food	0.829**	0.602*	0.921**	0.846**	0.789**
Noise	0.914**	0.898**	0.717**	0.638*	0.873**
Natural resources depletion	0.708**	0.804**	0.617*	0.837**	0.865**
Sustainability awareness	0.856**	0.763**	0.807**	0.764**	0.749**
* Significant at p<0.05. ** Significant at p< 0.001					

This result is consistent with what was reported by some previous studies that confirmed the existence of a positive correlation between knowledge and behavior. That is, between the three sides of the trend, the cognitive, emotional, and behavioral aspects. This is consistent with the model presented by psychologists, which emphasizes that the cognitive and emotional sides work to direct the behavioral side of the individuals, (Sitompul,2020).Awareness of sustainability, including perception, feeling, and knowledge of the dimensions of environmental pollution problems in terms of their causes, effects, and means of solving them, lies in its core positive, wise, and rational practices to protect the environment from pollution. Environmental awareness is related to its ability to form a sense of the problem, and its awareness leads to a sense of urgency.

The current study outcome is compatible with the findings of Qadir (2019) study, which indicates that the success of awareness programs is related to the ability to create a sense of the problem and its awareness, leading to a sense of urgency. Furthermore, Abd al-Masih (2019), which showed statistically significant differences in sustainability awareness of students in different educational stages before and after the implementation of sustainability awareness programs. Thus, there is a positive relationship between knowledge and awareness, and the attitudes and behaviors toward preserving the

environment. While the results of this study differed with some other studies that showed the opposite of that, and the importance of the impact of sustainability awareness in creating a change in the dynamics and behaviors of the individuals, indicating that the knowledge and information that individuals received will not help in changing individuals dynamics and attitudes to protect the environment, Pointed (Littlejohn, 2015) Pointing out that (80%) of those who received theoretical lectures on environmental preservation & sustainability did not have any impact on the dynamics & behaviors towards protecting the environment. This is because they did not develop a sense of urgency and awareness of it.

Table 9. Level of environmental sustainability awareness among the Saudi housewives

Sustainability variables awareness	Relative variables	Percentage	Order
Air pollution awareness	215	24.1	1
Food contamination awareness	241	27	2
Noise pollution	206	23.1	3
Natural Resource depletion	229	25.7	4
Total	891	100	

Table 10. Relative variables of taking the right decision to preserve the domestic environment

Taking decisions variables to preserve the domestic environment	Relative variables	Percentage	Order
Taking decisions against air pollution	229	24.3	3
Taking decisions against food contamination	244	25.8	2
Taking decisions against noise pollution	210	22.2	4
Taking decisions against resources depletion	261	27.6	1
Total	944	100	10

Several studies' results revealed the insignificance and usefulness of sustainability awareness and programs influencing students' attitudes and creating a positive change in their dynamics to protect the environment. And thus, there is no relationship between knowledge and individual's behavior (Al-Azhar, 2016; Hassanin, 2018). Abdul Hamid (2019) agreed with the results of these studies, where he indicated that knowledge is one thing, and practice and implementation is another, and that there is a big difference between saying, doing, knowing, and actually applying, and a weak relation associates them together. This means that the relationship between sustainability awareness and the individual's behavior to preserve the environment is fragile.

The fourth hypothesis, ''the difference in the relative measures between each variable of sustainability awareness and the actual practices performed by the Saudi housewives''. The relative measures of all the elements of sustainability awareness that was included in the research (air, food & noise pollution, and depletion of natural resources) and sustainability awareness as a concept. It was found that the level of sustainability awareness among the housewives' sample of the research. And the relative measures of all the variables of practices towards protecting the environment was found to determine the capacity level for practices towards protecting the domestic environment from pollution and its elements (awareness of sustainability for air, food, noise, depletion of natural resources, and awareness of the concept of sustainability as a whole) among the housewives Tables (9) and (10).

Awareness of environmental sustainability: It is evident from Table (9) that the ranking of the level of sustainability awareness of among the housewives came as follows: Food sustainability awareness came in first; secondly, the depletion of the natural resources, in the third place was the air pollution, and lastly noise pollution awareness. These results indicate an increase in awareness of sustainability in the area of awareness of food contamination. It also shows the high level of awareness of sustainability in the field of depletion and misuse of natural resources among the housewives subjected to the research.

The practices performed by the housewives to preserve the environment: Table (10) shows the ranking of practices towards protecting the domestic environment from pollution among the housewives as follows: resource exploitation came. First, the practices regarding food contamination came in second, then practices regarding air pollution, and lastly, practices on noise pollution. These results indicate the high degree of practices performed by Saudi housewives toward preserving the environment. This indicates the high level of administrative and consumption awareness among the Saudi housewives of the research sample. It also shows the high capability potentials to perform positive practices in the field of food contamination, which shows the high-level of attention these housewives are paying towards having healthy family members by avoiding the causes and sources of food contamination. This is when determining their nutritional needs, purchasing food commodities, preparing, cooking, and providing healthy meals to them, taking into account the methods of rationalizing food consumption. These results indicate that awareness of the concept of sustainability is related to wise practices to protect the environment. This applies to what was reported by previous studies, which

confirmed the existence of a positive correlation between knowledge and dynamics.

The fifth hypothesis "the share of independent variables (socioeconomic variables) with both awareness of sustainability and the actual ability to perform practices to preserve the environment from pollution among housewives as dependent variables according to the measures of regression coefficients and the degree of correlation with the coefficients". The percentage of participation of the independent variables (socio-economic level variables) with the dependent variable (sustainability awareness) and (the ability to make decisions towards protecting the environment) was calculated as a dependent variable among the housewives, according to the measures of regression coefficients and the degree of association with the dependent variable.

Table 11. Multiple regression analysis by step-forward method for independent variables (socioeconomic variables) with the dependent variable (sustainability awareness)

	Independent Variable	Dependent Variable	Relativity variable	Participation %	(F) Value	Significance	Regression factor	(T) Value	Significance
variable (sustainability awareness	1st step	Husband's education	0.851	0.725	73.816	0.1	0.545	8.592	0.1
	2nd step	Housewife's education	0.819	0.671	57.160	0.1	0.482	7.560	0.1
	3rd step	Housewife's age	0.772	0.595	41.209	0.1	0.398	6.419	0.1
	4th step	Income	0.740	0.548	33.923	0.1	0.348	5.824	0.1

Table 12. Multiple regression analysis in a step-forward method for independent variables (socioeconomic variables) with the dependent variable (practices towards preserving the environment)

Environmental sustainability variables	Independent Variable	Dependent Variable	Relativity variable	Participation %	(F) Value	Significance	Regression factor	(T) Value	Significance
Dependent variable Practices towards protecting the environment from pollution	1st step	Husband's education	0.882	0.778	98.084	0.1	**0.611**	**9.904**	0.1
	2nd step	Housewife education	0.794	0.631	47.928	0.1	**0.437**	**6.923**	0.1
	3rd step	Housewife age	0.757	0.574	37.683	0.1	**0.375**	**6.139**	0.1
	4th step	Income	0.725	0.526	31.037	0.1	**0.325**	**5.571**	0.1

Environmental sustainability awareness: It is evident from Table (11) that the husband's educational level was the first variable to be introduced in the regression analysis (first step), as the value of the participation rate was (0,725). This means that (72.5%) of the husband's educational level participates in raising the level of awareness of sustainability for the housewives, where the value of T (8,592) is statistically significant at the level of significance of (0.01). This means that the husband's educational level is one of the most important factors to raise awareness of sustainability among the housewives, with the positive impact it has on acquiring information and experiences through family relations and interactions between them

This is followed by the education of the housewives themselves (the second step) at a rate of (67.1%), where the value of the participation rate was (0.671) at a significant level of (0.01). This confirms that education contributes to gaining awareness of sustainability, to raise their level of awareness and sense of urgency towards the environmental pollution, in terms of their causes, effects, and means of solving them. Then came in the age of the housewives (the third step) at a rate of (59.5%), Where the value of the participation rate was (0.595) at a level of significance (0.01), which means that the age of the housewives is one of the factors affecting awareness of sustainability. That is, the higher the age of the housewife, the higher the level of awareness of sustainability she possesses. Then came in the husband's profession (in the fourth step). The last at a rate of (54.8%), where the value of the participation rate was (0.548) at the level of significance (0.01), this means that the husband's profession is one of the factors affecting the awareness level of the housewives, with the distinct educational level associated with it. Thus, its reflection on the level of awareness of sustainability.

The ability to take decisions towards protecting the domestic environment from pollution: Table (12) shows

that the educational level of the wife was the first variable to be introduced in the regression analysis (the first step), as the value of the participation rate was (0.778) meaning that (8.77%) of the wife's educational level participates in raising awareness of sustainability, for the housewives, where the value of T (9.904) is statistically significant at the level of significance of (.010), which means that the educational level of the wife is one of the most important factors affecting the improvement of her ability to carry out these practices to protect the domestic and external environment. This result is aligned with the findings of the study of (Resurrección, 2013). Which indicated a correlative relationship between the wive's perception of the problem of pollution and planning the available resources; secondly the age of the housewives (the second step) is at (63.1%), where the value of the participation rate was (0.631) at a significant level of (0.01),

This confirms that age contributes to gaining experiences related to the actual practices performed to protect the environment. Then the husband's education came in (the third step) at a rate of (57.4%), where the value of the participation rate was (0.574) at a level of significance (0.01). This means that the husband's education is one of the factors affecting the actual ability to perform positive practices to protect the environment, then came the monthly income (fourth step), meaning that the monthly income is one of the factors that affect the level of practices as well, with the financial capabilities that help the housewives in choosing the various means and methods to alleviate environmental pollution.

CONCLUSION

The current study highlights the existence of a positive significance correlational between awareness of the concept of sustainability in the elements (air, food, natural resources exploitation and rationalization of their consumption, and noise) and the variables of practices towards preserving the environment. There were significant differences between the awareness level and the variables of practices to protect the environment among the working and non-working housewives, as well as between sustainability awareness in the elements (air, food, natural resources exploitation and rationalization of their consumption, and noise) and the variables of practices to preserve the domestic environment among the housewives of the research sample, according to their different educational levels.

Also, there was a positive significance correlation between awareness of the concept of sustainability in the elements variables of (air, food, natural resources exploitation and rationalization of their consumption, and noise) and between practices to protect the domestic environment from air, food, noise pollution and depletion of resources, while the results showed that there is no relationship between them and the number of family members, and also showed the level of sustainability awareness among the housewives to protect the environment, came as follows: awareness of food contamination came in first, then depletion of the natural resources is ranked second, then awareness of air pollution ranked third, then awareness of noise pollution came fourth and last. And that the ranking of the actual level of ability to perform positive practices to protect the environment among the housewives of the research sample, in all its variables came as follows: the ability to take decisions towards protecting the domestic environment and resource exploitation came in first, food contamination came in second, then air pollution ranked third, and then noise pollution came in fourth and last.

Recommendations: Based on the research results, it is recommended that it should raising sustainability awareness through women and family programs using all media and various social media platforms, on the other hand proposing appropriate and easy-to-implement solutions in the environment. Focus on aspects of environmental awareness in the educational curriculums in all different educational stages. Raising awareness will enforce the change in the behavioral habits & family dynamics to preserve the domestic and environment in general, and develop a strategy to protect the environment from pollution in an integrated system that leads to interaction and integration between the environmental advocates, family members, and the whole community to face pollution problems in the Saudi society.

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Elucidation of Antifungal, Antioxidant and Anticholesterol Activity of Efficiency of Fungal Statin Isolated from *Aspergillus tamarii*

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ABSTRACT

Statins are drugs that lower the level of low-density lipoprotein (LDL) cholesterol levels as statins involve as potent inhibitors of cholesterol synthesis in blood. The use of statins in the medical field has enormous applications. Among few recent applications, Statins used in hospitals are proved to lower the risk of mortality among individuals effected with corona virus, its effective in protection against the neurodegenerative disorder, local application of statin have proved to repair bone.The present study was delineated to isolate the fungal strains and to screen statin production analyzed by growth inhibition of *Candida albicans* and *Aspergillus fumigatus*, further applied to evaluate the antioxidant and anticholesterol activity of fungal statin isolated from *Aspergillus tamarii*. This report depicts the antifungal activity of isolated fungal statin was done by well diffusion method which showed susceptibility of *Candida sp*. against the fungal statin, antioxidant potential of fungal statin was observed by DPPH (2,2 - diphenyl- 1- picrylhydrazyl) radical scavenging assay that proved to have increasing inhibition percentage with increasing concentration from 25µg/mL to 100µg/mL. Further, the anti-cholesterol analysis was done using male albino rats having high cholesterol diet proved that the high fat diet had adverse effect on the liver, while total bilirubin showed only marginal increase when compared to normal rats. Therefore, this study depicts an unprecedented work of fungal statin from *Aspergillus tamarii*, showing good potential to be used as antifungal, antioxidant and cholesterol lowering agent. Therefore, these natural statins could be used as a replacement for chemical drugs with no adverse side effects.

KEY WORDS: ASPERGILLUS TAMARII, ANTIFUNGAL, ANTIOXIDANT, ANTI-CHOLESTEROL.

ARTICLE INFORMATION

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INTRODUCTION

Secondary metabolite production, a hallmark of filamentous fungi, is an inflating area of research for the *Aspergilli*. A variety of biologically active compounds are produced by fungi, mainly by the polyketide biosynthetic pathway. Fungal polyketides comprise a very large and structurally diverse group and many display important biological properties such as antibiotic activity and other related pharmacological properties (Bedford et al., 1995). Among the fungal metabolites, statins (anticholesterol compounds) are considered as the most important class of secondary metabolites produced by the polyketide pathway. Statins shows anti-fungal effects (Tavakkoli et al., 2020).

Statins are a class of molecules with a polyketide structure, obtainable by secondary fungal metabolism, which can inhibit HMG- CoA (hydroxyl methyl glutaryl - coenzyme A) reductase activity. Thus the mechanism involved in the control of endogenous cholesterol levels by Statins ; makes the molecules suitable for therapeutic use (Alberts et al., 1980; Endo, 1985a; Alberts, 1988; Farnier and Davignon, 1998; Stein et al., 1998; Furberg, 1999; Maron et al., 2000; Chong et al., 2001; Tobert, 2003). Initially statins were discovered in fungi and for many years fungi were the sole source for the statins (Subhan et al., 2016).

Lovastatin is a naturally occurring statin obtained from different genera and species of filamentous fungi. Fungal strains including *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopolariopsis, Doratomyces, Phoma, Phythium, Gymnoascus, Hypomyces* and *Pleurotus* have been reported as lovastatin producers. Of many statin molecules, lovastatin and mevastatin are produced by the fungal species, while other statins like rosuvastatin, simvastatin, pravastatin, fluvastatin, atrovastin, cerivastatin are produced semi- synthetically from lovastatin (Tobert, 2003, Chakravarti and Sahai, 2004). *Pleurotus sp*. and its related strains produce higher lovastatin (Srinu et al., 2010). Recent research has focussed on the metabolic regulation of MK/lovastatin synthesis and its evidence shows that the combination of extracellular and intracellular factors is an important significance essential for MK/lovastatin metabolism (Zhang et al., 2020).

Research with fungal metabolites identified a series of compounds with potent inhibiting properties for this target enzyme HMG- CoA reductase, from which lovastatin was selected for clinical development. Cholesterol synthesis reduction by lovastatin was confirmed in cell culture, animal studies and in humans. The subsequent reduction in circulating total and low-density lipoprotein (LDL) cholesterol has also been demonstrated in animals and humans. Major mechanism of LDL clearance from the circulation were caused by hepatic LDL receptors, which proves to be the major mechanism, further research in animals has confirmed that these declines in cholesterol are accompanied by an increase in hepatic LDL receptor activity. Statin effectively diminishes endogeneous cholesterol synthesis providing useful therapeutic properties for patients with hypercholesterolemia (Morris et al., 1993; Jenkins et al., 2005).Another interesting property of statins is that they have an effective antifungal potential against both yeast and filamentous fungi; furthermore they can be combined with clinically used antifungal agents (Galgoczy et al., 2011).

Several *in vitro* and *in vivo* studies have been carried out targeting the antioxidant potential of various types of natural and synthetic statins such as atorvastatin, (Wassman et al., 2002), pravastatin (Alanazi, 2010), fluvastatin and simvastatin (Franzoni et al., 2003).Fungi have been identified as the new sources of antioxidants due to wide production of secondary metabolites (Arora and Chandra, 2010). Recent studies reported that the preparation and characterization of nano statins using oyster mushroom (*Pleurotussajorcaju*), reduces toxicity and enhance efficacy for treatment of cardiovascular disease (Mehra et al., 2020).

MATERIAL AND METHODS

Isolation of fungal strains: The fungal isolates to be used in the study were collected from different environment sources which included sources like coffee powder, corn, coconut and cotton seeds. Coconut and corn previously infected with fungus were taken. All the samples which were incubated were further inoculated onto potato dextrose agar and incubated at room temperature until growth was observed. Cultures were subcultured to obtain pure cultures.

Screening of statin production analyzed by growth inhibition of *Candida albicans* and *Aspergillus fumigatus*: *Candida albicans* and *Aspergillus fumigatus* strains were grown on potato dextrose broth containing various concentrations of 50mg/mL,100mg/mL and 250mg/mL of cholesterol and incubated at 35˚C for 48hours for 5 days. After 5 days of growth, the cultures were then inoculated with YEPD agar to which 100µl of aqueous extract of fungal statin from *Aspergillus tamarii* was added.

Application of the isolated fungal statin: Antifungal activity of isolated fungal statin: The anti-fungal activity of the isolated statin was compared with commercially available statins which include simvastatin, rosuvastatin, atorvastatin and lovastatin. The cultures include *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Fusarium, Rhizopus, Excerohilumsp.,Candidaalbicans, Candida albicans* clinical strains, *Candida krusei,Candidaglabrata, Candida tropicalis*. The fungal cultures were maintained on potato dextrose agar at 28˚C.

Anti-oxidant analysis of isolated fungal statin: The purified fungal statin and commercial statin were further evaluated for their antioxidant potential. Commercial statin and the isolated compound was dissolved in ethyl acetate and stored at 4˚C. 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml of the purified statin were subjected to standard

antioxidant assays such as DPPH radical scavenging assay. The standard antioxidant assays were checked for percentage inhibition or scavenging activity by the formula, DPPH inhibition percentage had been calculated using the formula.DPPH inhibition (%) = [Control absorbance – Test absorbance] x 100.

Table 1. Components of the high cholesterol diet administered to albino rats

Component	Measured amount (g)
Wheat flour	15
Roasted Bengal Flour	58
Groundnut Flour	10
Milk Powder	5
Powdered cashew nut	4
Salt	4
Casein	4

Table 2. Grouping of animals for experimental study *in vivo*

Groups	Experimental Design
Group I / Normal	Served as normal control rats where the diet administered was the normal diet without the cholesterol products.
Group II	Rats fed with 10g of high cholesterol diet per kg Body Weight (BW) of the rat for 45 days.
Group III	Rats fed with 10g of high cholesterol diet containing per kg of BW of the rat for 45 days along with 2mg of commercial statin per kg BW
Group IV	Rats fed with 6mg purified fungal statin from solid state fermentation in addition to 10g of high cholesterol diet per kg BW of the rat for 45 days
GroupV	Rats fed with 6 mg purified fungal statin from submerged fermentation in addition to 10 g of high cholesterol diet per kg BW of the rat for 45 days

Anticholesterol analysis: Male albino rats study was performed using high cholesterol diet, where high cholesterol products were supplemented with the commercial feed (Table:1). 2mg of commercial statin tablet and 6mg of lyophilized purified fungal extract was dissolved in 1ml sterile water, was administered on all days mixing 1ml with feed. Feeding was continued for about 45 days. After one week of adaptation, the animals were divided into 5 groups with 3 animals in each group. The control animals were fed with normal diet (Table:2). At the interval of 15days the body weight was observed. The weight at after 7days of the experiment was taken as the initial weight. After 45days, the rats were deprived with food overnight and the blood was collected by cardiac puncture. The serum was collected from blood, screened for cholesterol levels and liver marker enzymes.

Statistical analysis: The data was analysed using statistical package for social sciences (SPSS) 17.0. Mann Whitney U-test was performed to find the significance of the test results obtained as the variables were independent and the sample size was less than 10 (n=3).The significance value was considered at two measure of 95% ($p < 0.05$) and 99% ($p<0.01$) were determined to be statistically significant.

RESULTS AND DISCUSSION

Aspergillus section and Flavicontains a number of different fungal species which are well known for their production of secondary metabolites. Most of these secondary metabolites are promising candidates for human use as the natural replacements for artificial chemical compounds. The objective of the study was to isolate and purify fungal statin from *Aspergillus tamarii* and determine its potency as an anticholesterol agent, antioxidant, antifungal agent and anticancer agent. In this study, we have isolated 15 fungi from various environmental sources, out of which 14 were found to be *Aspergillus flavus* and one was found to be *Aspergillus tamarii*. The aflatoxin analysis revealed that the isolated strain of *Aspergillus tamarii* was non- aflatoxigenic. Osman et al.,(2011) screened twenty three fungal isolates and tested for their ability to produced lovastatin.

Screening of effects of statins on the growth of *Aspergillus fumigatus* were investigated on solidified minimal media. *Aspergillus fumigatus* exhibited robust growth with the production of conidia after 4days at 31˚C. In the presence of statins, there was growth inhibition in regular *Aspergillus fumigatus* strains. Strains were initially grown on various concentrations of cholesterol with PDA and the spores were plated with statin incorporated solidified PDA. The cultures exposed to higher concentrations of cholesterol were shown higher growth in the presence of statin. (Table.3).

Antifungal activity of isolated fungal statin: The antifungal activity of the isolated fungal statin was analysed against different fungi along with commercially available statin. Antifungal susceptibility of *Candida sp.* was confirmed against the isolated fungal statin (Table: 4). Galgoczy et al., (2011) studied the in vitro antifungal activity of statins against yeast and filamentous fungal isolates including *Candida albicans, Candida glabrata, Candida krusei,Candidaparapsilosis, Candida tropicalis, Rhizopus sp., Aspergillus flavus*

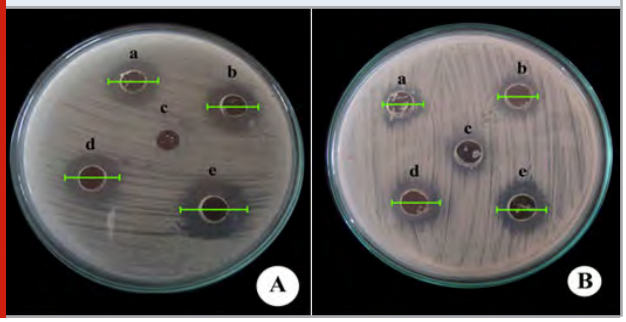
and *Aspergillus fumigatus*. The inhibitory potentials of statins were studied in the range 0.25 - 128 µg/ml by broth microdilution.

In study simvastatin (8µg/ml) displayed the strongest antifungal activity followed by fluvastatin (25- 128 µg/ml), atorvastatin (128 µg/ml), rosuvastatin (128 µg/ml) and lovastatin (5-64 µg/ml) against yeast while antifungal activity of statins against filamentous fungi showed fluvastatin (2µg/ml) to be most potent followed by rosuvastatin (8µ/ml), simvastatin (6.25 µg/ml), lovastatin (25 µg/ml) and atorvastatin (>128 µg/ml). In our study rosuvastatin, atorvastatin, simvastatin, lovastatin, showed potency at (>100 µg/ml), while fungal statin showed inhibition at (300µg/ml) against Candida sp. and filamentous fungi including *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Rhizophus sp., Excerohilum sp.* and *Fusarium sp.* did not show any inhibition against fungal statin and commercial statin.

Table 3. Bioassay using *Candida albicans* (MTCC183) showing diameter of inhibition zone using fungal extract on comparison with commercial statin

S.No.	Concentration of commercial statin (µg/ml)	Diameter of inhibition zone using commercial statin (cm)	Concentration of fungal statin (µg/ml)	Diameter of inhibition zone using fungal statin (cm)
1	50	1.8	50	1.2
2	75	2.1	75	1.6
3	100	2.6	100	1.9
4	125	3.2	125	2.8
C	Control (ethyl acetate)	-	Control (ethyl acetate)	-

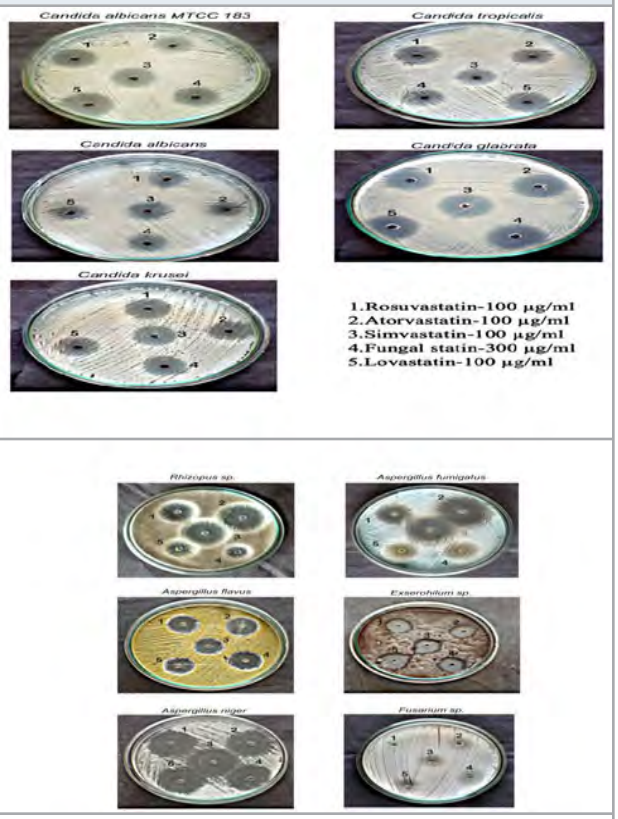
Plate 1: Diameter of zone of inhibition using Candida albicans(MTCC183) against(A) commercial statin and (B) fungal extract



a-50 µg/ml; b-75 µg/ml; c- control (ethyl acetate); d- 100 µg/ml; e-125 µg

Antioxidant activity of isolated statin: The DPPH activity of the statin compound when compared to the commercial antioxidant BHT. The concentration dependent comparison confirmed extracted statin to possess antioxidant activity (Figure 3). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) as commercial antioxidants now play a role in screening of antioxidant potential in metabolites and biopharmaceuticals extracted or isolated from plants or microbes (Ozusaglam and Karakoca, 2013). In our study, DPPH radical scavenging activity of fungal statin showed similar activity when compared to standard statin tablet although there was a significant increase in the antioxidant activity by BHT (the commercial antioxidant). The variation of the fungal statin with respect to enzymatic antioxidants is largely dose dependent. An increase in the antioxidant potential of fungal statin was

Plate 2: Antifungal activity of lovastatin and fungal statin



1 – Rosuvastatin (100 µg/ml); 2 – Atorvastatin (100 µg/ml); 3 – Simvastatin (100 µg/ml); 4 – Fungal statin (300 µg/ml); 5 – Lovastatin (100 µg/ml)

observed with increase in the concentration from 25 µg/ml to 100 µg/ml. The lower yet statistically significant antioxidant potential confirmed by standard DPPH and enzymatic assays reveal the efficacy of fungal statins as an antioxidant agent.

Anticholesterol analysis: At the end of 45days of treatment, thebody weight of all groups treated and normal were compared and represented as mean ± standard error mean (SEM). A gradual increase in the body weight of the normal population was seen. On comparison, there was a spiked increase in the body weight in group II, group III, group IV and group V where the diet was treated with additional fat products such as casein and cashew nuts as shown. (Table 4). The body weight of the animals treated with high fat diet ($p<0.05$) showed a significant increase in the body weight was seen after 30days and 45days of feeding when compared to normal animals. The levels of serum lipid profile, total cholesterol (TC), triglycerides (TG) , LDL-C, VLDL-C and HDL-C in normal and drug treated rats are presented in (Table 5).

Table 4. Antifungal activity of different types of statin against various fungal species

S.No	Fungal cultures	Fungal statin (300µg/ml)	Lovastatin (100µg/ml)	Simvastatin (100µg/ml)	Atorvastatin (100µg/ml)	Rouvastatin (100µg/ml)
1	*Candida albicans* MTCC 183	+	+	+	+	+
2	*Candida albicans*	+	+	+	+	+
3	*Candida krusei*	+	+	+	+	+
4	*Candida glabrata*	+	+	+	+	+
5	*Candida tropicalis*	+	+	+	+	+
6	*Aspergillus niger*	+	+	+	+	+
7	*Aspergillus flavus*	+	+	+	+	+
8	*Aspergillus fumigatus*	+	+	+	+	+
9	*Rhizopus*	+	+	+	+	+
10	*Exserohilum*	+	+	+	+	+
11	*Fusarium*	–	–	–	–	-

'+'-Inhibitory effect '–' - No inhibition

Table 4. Comparison of body weight through the period of 45 days at the intervals of 15 days

Treatment (mg/kg of BW)	Changes in Body Weight (mg)			
	0day (Initial)	15 days	30 days	45 days
Normal	155±1.73	159.33 ± 0.57	163.33 ± 0.57	165.66± 0.57
Group II (High Fat Diet)	156 ± 3.46[b]	179 ± 4.58[a]	186.33 ± 2.51[a]	190.33 ± 1.52[a]
Group III (High Fat Diet + Commercial Statin)	156.66 ± 2.08[b]	184 ± 4.58[a]	174.66 ± 1.52[a]	171.33 ± 1.52[a]
Group IV (High Fat diet + Purified fungal statin from solid state)	158.33 ± 2.08[b]	185.33 ± 2.51[a]	170.33 ± 4.5[a]	165.33 ± 2.08[b]
Group V (High Fat diet + Purified fungal statin from submerged)	156.66 ± 1.5[a]	183 ± 1[a]	167 ± 2.64[b]	163.66 ± 3.2[b]

All values have been mentioned in mean ± SD; a – n=3 observations at $p<0.01$ on comparison with normal group, b – n=3 observations at $p<0.05$ on comparison with normal group

Statin induced rats showed significant decrease in serum HDL-C profiles when compared with normal rats. There was also decrease in the levels of cholesterol and triglycerides in both the drug treated and fungal statin treated rats when compared to high fat diet group. LDL levels were found to be significantly higher in the groups treated with commercial statin and fungal statin when compared to normal controls. The specific liver markers such as bilirubin, SGOT, SGPT, and alkaline phosphatase in serum in all five groups studied. (Table 6) Significant increase in the levels of SGOT, SGPT and alkaline phosphatase in group II when compared with group I.

On comparison with group II, there was a significant decrease in SGOT, SGPT, and alkaline phosphatase values in group III, group IV and group V. This showed that high fat diet had negative effect on the liver, while the total bilirubin showed only a marginal increase when compared to normal rats.

Table 5. Effect of purified statin and commercial statin on serum lipid profile of normal and induced adult male albino rats

Group	TC	TG	HDL-C	LDL-C	VLDL-C	CHO/HDL	LDL/HDL
I	62.3±1.1	56.8±3.2	26.6±1.52	24.9±1.4	11.2±0.64	2.3±0.644	0.9±0.1
II	85.8±4.7^{a}	116.6±1.2^{a}	32.6± 1.5^{b}	29.5±1.44^{b}	23.5±2.6^{a}	2.62±0.03^{a}	0.9±0.5^{b}
III	68.7±1.5^{d}	46±8.7^{e}	22.6±2.51^{e}	36.8±3.40^{e}	9.2±1.74^{d}	3.05±0.38^{d}	1.6±0.3^{e}
IV	63.5±3.04^{ec}	34±2.64^{ec}	12.3±2.5^{dc}	39.7±1.16^{dc}	6.8±5.2^{dc}	5.26±0.87^{dc}	3.7±0.7^{dc}
V	65.01±2^{d}	41.66±4.5^{bd}	15.66±3.8^{ad}	41.01±3.8^{bd}	8.33±0.9^{ad}	4.31 ±1	2.74±0.86

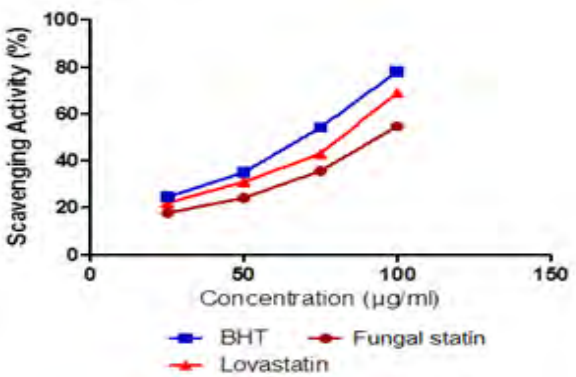
Each value is given in Mean±SEM; a – n=3 observations at p<0.01 on comparison with group l, b – n=3 observations at p<0.05 on comparison with group I, c – observations with p<0.05 on comparison with group III, d – n=3 observations at p<0.01 on comparison with group II, e –, n=3 observations at p<0.05 on comparison with group II

Table 6. Effect of purified statin obtained on submerged and solid state fermentation and commercial statin on liver marker enzymes *in vivo*

Groups	Total Bilirubin	SGOT	SGPT	Alkaline Phosphatase
I	0.3±0.1	75.4±5.1	24.8±2.5	120.25±1.93
II	1.1±0.1	86.6±7.63^{a}	61.3±3.5^{b}	175±5^{a}
III	0.4±0.1	72.4±2.25^{c}	23.5±2.21^{c}	120.3±3.06^{c}
IV	0.4±0.1	74.9±1.5^{c}	30.4±2.4^{c}	121.8±1.2^{c}
V	0.6±0.1	75.2±2.1^{c}	28.8±1.48^{c}	122.7±2.5^{c}

Each value is given in Mean±SEM; a – n=3 observations at p<0.01 on comparison with group l, b – n=3 observations at p<0.05 on comparison with group I, c– n=3 observations at p<0.01 on comparison with group II.

Figure 1: Antioxidant activity of isoalted statin



Tanideh and Badiei, (2013) studied the effect of simvastatin and garlic on lipid profile and liver marker enzymes in rats fed with normal and fat rich diet. Simvastatin significantly reduced the total cholesterol, triglycerides and LDL broth on normal diet and fat rich diet. HDL increases significantly in simvastatin treated rat. No significant changes were detected in ALT and AST levels in different groups. Rajasekaran and Kalaivani, (2011) studied the hypolipidemic and antioxidant activity of aqueous extract of *Monascus purpureus* fermented Indian rice in high cholestrol diet fed rats. On in vivo evaluation of anticholestrolemic activity, the plasma total cholestrol, triglycerides, LDL and VLDL significant declined when compared to the high cholesterol fed rats. Recently Yuliana et.al (2020) have reported the fermentation and determination of Anticholesterol Monakolin K from different isolates of *Monascus purpureus*.

CONCLUSION

This is the first report on the isolation of fungal statin from Aspergillus tamarii. Statin is produced as a part of the polyketide pathway during fungal metabolism. Statins have a variety of biological effects which include anticholestrolemic, antimicrobial and antioxidant properties. This study was designed to isolate the fungal statin from *Aspergillus tamarii*, that has showed good potential to be used as an antifungal agent, anticancer agent, antioxidant agent and a cholesterol

lowering agent. These natural statins could be used as a replacement for the chemical drugs that come along with various side effects.

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Conflict of interest: The authors declare no conflicts of interest.

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Limnochemical Characterization of Tidal Stretch of the River Hooghly in West Bengal

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ABSTRACT

River Ganga is one of the most important river systems in India and it is regarded as the most sacred river of this country. The river bifurcates near Murshidabad district of West Bengal – one distributary flows eastward and enters into the Bangladesh as "Padma" and the other branch flows further southward direction through the state of West Bengal (India) with the name "Hooghly" or "Bhagirathi-Hooghly". Nowadays this holy river is facing tremendous pressure from rapid industrialization, urbanization and different agro-industrial developments in the catchment areas. The basic objectives of our present study were to investigate the seasonal variation of different limnochemical characteristics of river Hooghly at some predesigned stations and also to assess the effects of pollution on spatial and seasonal point of view. In the two years long (from March-2017 to February-2019) study we investigated the riverine water quality on the basis of eight (08) limnochemical parameters (Water Temperature, pH, Hardness, Dissolved Oxygen, Biological Oxygen Demand, Chemical Oxygen Demand, Total Nitrogen and Total Phosphorus). The acquired dataset were analysed using ANOVA and multivariate statistical analysis like PCA to obtain the major factors regulating the water quality and to measure the spatiotemporal variation. This investigation would be helpful to understand the present status of limnological characteristics and to frame out the control strategy for the upcoming eras. .

KEY WORDS: LIMNOCHEMICAL CHARACTERISTICS, RIVER HOOGHLY, SEASONAL VARIATIONS, PCA.

INTRODUCTION

We all know that the "water" is called as "life" as it sustains the lifeline of the whole organism in the planet and that's why our planet is known as "Blue Planet". River Ganga is regarded as most significant river system in India due to its water availability round the year. Besides this river plays a key part in maintaining the growth of civilization and economy of Indian people (Paul and Sinha, 2013). In one word, River Ganga is the lifeline of about 44% of country's population (Chaudhary et al., 2017). In the diversified cultural ethos of India, the holy river Ganges occupies a unique position. It is regarded as one of the most "sacred river" not only in India but also in the entire world. This river has been deeply admired by the millions of people of India since ancient times. River Ganga holds almost 25 per cent of total Indian water resources (Rahaman, 2009).

The Ganga river basin (Fig.1) is the chief and largest river basin in India (Agarwal, 2015). It is considered as one of the most densely populated areas in the world and the basin comprises about 300 million people including three countries, namely India, Bangladesh and Nepal

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(Das and Tamminga, 2012; Gopal, 2000). This river basin encompasses a large area between 22°30′ to 31° 30′ N latitudes and 73° 30′ to 89° 00′ E longitudes. The river Ganges rises from the Gaumukh ice cave (Lat. 30° 55'N, Long. 79° 07'E) of Gangotri Glacier located on the southern slope of Garhwal Himalaya at an altitude of 4100 meters.

The main river starts its journey from Gaumukh glacier as "Bhagirathi", and then it flows further downward and joins with river "Alakananda" at the place Devprayag. After that the joint flow of Bhagirathi and Alakananda is named as river "The Ganga". It runs through different state of India and finally meets with Bay of Bengal at the place Gangasagar in West Bengal. The total length of Ganga River is about 2525 kilometers (CPCB, 2013). River Ganga gets its way to the West Bengal with the name of river Bhagirathi through Rajmahal Hills of the state Jharkhand and then it streams about 80 kilometers downward up to Farakka in West Bengal. The main stream of Ganga is known as river Bhagirathi from Mithipur village of Murshidabad district of the state West Bengal. Then the river flows about 500 kilometres towards south to meet finally the Bay of Bengal at Gangasagar. The same stream is further named as "River Hooghly" from Nabadwip to Gangasagar stretch (about 280 km) and this entire stretch is tidal in nature (Rudra, 2016; State of Environment Report West Bengal, 2016).

The complete path of River Ganga can be subdivided into three different zones (GAP annual report, 2015; Dutta et al., 2020) – a) Upper stretch, which starts from Gamukh glacier to Haridwar (total length about 294 kilometres) (b) Middle stretch, which starts from Haridwar to Varanasi (total length about 1082 kilometres) (c) Lower stretch, which starts from Varanasi to Gangasagar of West Bengal where it meets Bay of Bengal (total length about 1134 kilometres). Gangasagar is also known as Sagar Island and this area is subjected to heavy anthropogenic pollution load during January of every year on the occasion of Holy Bath (Bonnail et al., 2019). The Ganga river basin is the one of the most densely populated regions of the earth and it is the biggest groundwater repositories (Pal et. al., 2020).

Figure 1: Ganga river basin (adopted from National River Conservation Directorate, MOEF, 2009)

But this huge water resource is being polluted from various sources in successive years. From last few years Government of India had taken a variety of strategies like Ganga Action Plan (GAP) and founded National Ganga River Basin Authority (NGRBA) to rejuvenate the river water, but its success rate is quite low (Chaudhary and Walker, 2018). The main objectives of this research paper are to assess the vital physico-chemical parameters of Hooghly (The Ganga) river water mainly in the tidal stretch from Nabadwip to Gangasagar in West Bengal and to analyse its water quality through the time period.

Figure 2: Lower stretch of River Hooghly (The Ganga) showing the sampling stations (modified after Alam, 2020)

MATERIAL AND METHODS

Climatic conditions of study areas: The climatic conditions of River Ganga (Bhagirathi-Hooghly) basin of southern part of West Bengal vary from Humid to arid, it experiences well-defined seasons in a year. For this study we categorized the study period into 3 different seasons namely Pre-monsoon (March-June), Monsoon (July-October) and Post monsoon (November-February).

Sampling stations: Five sampling stations were selected (Fig. 2), along north-south direction of the tidal stretch of the river covering a length of about 250 km. Selection of sampling stations were based on variability in geo-physical environment, varied nature of anthropogenic activities, land use pattern and several nonpoint and

point sources of pollution. Details of the sampling stations are given below.

1. **Nabadwip or G-1(23°24'N & 88°22'E):** This sampling site is located near Gouranga Setu (bridge) in the Nadia district of West Bengal. Here ecological stresses are agricultural runoff, ferry service, bathing, washing clothes, domestic sewage etc.
2. **Naihati or G-2 (22°53'N & 88°24'E):** This sampling site is located in the North 24Parganas district near the Lichubagan ghat. Ecological stresses are industrial effluent from Keshuram Rayon, Tribeni tissue paper mill and Bandel thermal power station, besides that bathing, ferry service, domestic sewage etc. also affect the quality of the riverine water.
3. **Dakhineswar or G-3 (22°39'N & 88°21'E):** This sampling site is located in the North 24 Parganas district of West Bengal. This site is under the purview of Kolkata Metropolitan Development Authority. Famous Dakhineswar Kali temple is situated in the vicinity of this sampling station. Flowers, food particles and remnant of puja materials are disposed here very often. Here bathing, washing clothes etc. deteriorate the water quality of this holy river.
4. **Gardenreach or G-4 (22°33'N & 88°17'E):** This sampling station is located in the south-western part of the Kolkata metropolitan area. Here ecological stresses are domestic sewage, industrial waste etc.
5. **Gangasagar or G-5 (21°47'N & 88°03'E):** This is located in the South 24 Parganas district of West Bengal. Bathing, fishing, tourist activities are evident here.

Table 1. Limnochemical profile of water at different sampling stations (PRM=pre-monsoon, MON=Monsoon, POM=Post-monsoon) from Mar-2017 to Feb-2018.

Stations	Seasons	Water Temperature (°C)	pH	Hardness (mg/l)	DO (mg/l)	BOD (mg/l)	COD (mg/l)	Total Nitrogen (mg/l)	Total Phosphorus (mg/l)
Nabadwip (G1)	PRM	30.875±5.07	8.03±0.42	155.25± 14.22	9.3±2.09	2.39±0.65	10.05±1.49	0.76±0.62	0.021±0.006
	MON	28.625±0.47	7.3±0.32	110.25± 29.30	6.48±0.55	3.51±1.17	11.87±2.50	1.67±1.09	0.076±0.010
	POM	22.625±4.49	8.25±0.21	131.5± 25.51	7.5±0.76	3.36±1.51	15.6±4.10	1.04±0.53	0.052±0.022
Naihati (G2)	PRM	23.12±1.70	7.98±0.33	163.5± 15.67	8.7±2.00	2.03±1.45	14.10±5.23	0.82±0.633	0.06±0.036
	MON	30.5±0.62	7.39±0.05	107.75± 37.31	6.05±0.59	3.63±0.73	14.58±3.89	1.83±1.50	0.094±0.057
	POM	27.25±2.44	8.25±0.23	121.75± 23.62	6.27±1.60	3.45±1.19	13.27±6.55	0.757±0.379	0.12±0.096
Dakshineswar (G3)	PRM	31.45± 0.97	8.0 ± 0.49	108.69± 11.62	6.13±0.31	4.48±0.48	12.34±3.81	0.45±0.14	0.06 ± 0.04
	MON	28.42 ± 0.89	7.22±0.58	92.28± 5.41	4.8 ± 0.45	4.83±0.54	17.79±1.52	0.95±0.16	0.12 ± 0.07
	POM	24.32± 2.69	7.32 ± 0.55	86.02± 7.61	5.39±0.55	3.99±1.65	9.97± 1.11	0.91 ± 0.10	0.09± 0.03
Garden Reach (G4)	PRM	31.39 ± 3.16	7.7 ± 0.63	105.06± 13.78	5.32±0.56	4.80±2.20	9.30 ± 2.72	0.73± 0.18	0.078± 0.05
	MON	30.02± 2.25	7.62±0.35	100.04± 10.29	4.98±0.71	5.52±1.84	13.07±3.61	1.33 ± 0.63	0.05± 0.04
	POM	21.10 ±2.63	7.67±0.47	95.48 ± 13.82	5.93±1.23	4.53±1.04	7.78 ± 2.88	1.41± 0.59	0.08± 0.04
Gangasagar (G5)	PRM	29±4.83	7.39±0.36	380.25± 33.27	7.12±0.94	2.86±1.80	27.32±10.62	0.665±0.24	0.06±0.021
	MON	27±3.69	7.24±0.18	335± 30.81	5.97±0.59	3.66±1.46	22.03±8.59	0.525±0.10	0.052±0.027
	POM	25.75±4.54	7.76±0.34	365.25± 60.25	6.06±0.64	2.93±1.58	26.83±11.47	1.295±0.39	0.077±0.068

Water Analysis: For the analysis of different limnochemical parameters monthly sampling of water from all the stations was done for two years (from March-2017 to February-2019) and monthly collections of the water samples were prepared from each of the sampling stations between 7 am to10 am. During each sampling water samples are collected in three replicates from surface, column and bottom of each sampling station and average values of all observations are taken into consideration (Debnath et al., 2013). Water samples are also collected

twice along the opposite river banks and once from the middle of the width. Average of three samples has been recorded as monthly data for each parameter.

Estimation of various physico-chemical parameters like water temperature, pH, DO were done on the spot. Water temperature and pH was recorded with the help of mercury bulb thermometer and portable pH meter (Hanna, model) respectively. DO of the water samples were analysed by Sodium azide modification of Winkler's method (Ghosh & Panigrahi, 2018). Rest of the physico-chemical parameters of water samples collected from different study sites were analysed in the laboratory within 24 hours following the standard methods (APHA, 1985; APHA, 2005; APHA, 2012; Chattopadhyay, 1998).

Statistical analysis: The limnological data of the two year study period was assembled for three seasons (by taking mean values of each parameter for four months of respective season) and assessment was done for seasonal variations, viz., Pre-monsoon (from March to June), Monsoon (from July to October) and Post-monsoon (from November to February). Mean, Standard Error of Mean and One- Way ANOVA for various parameters for three seasons and multivariate statistical analysis PCA (Principal Component Analysis) were performed using SPSS 18.0 for Windows, MS Excel 2010 and PAST ver. 4.0.

Table 2. Limnochemical profile of water at different sampling sites (PRM=pre-monsoon, MON=Monsoon, POM=Post-monsoon) from Mar-2018 to Feb-2019.

Stations	Seasons	Water Temperature (°C)	pH	Hardness (mg/l)	DO (mg/l)	BOD (mg/l)	COD (mg/l)	Total Nitrogen (mg/l)	Total Phosphorus (mg/l)
Nabadwip	PRM	32.4±4.03	8.29±0.26	148.4±18.28	8.40±1.29	2.29±1.29	11.25±4.20	1.306±0.60	0.06±0.05
(G1)	MON	25.68±1.44	7.70±0.51	114.12±31.49	6.15±1.33	3.93±1.42	12.00±4.93	0.69±0.38	0.065±0.019
	POM	27.62±4.11	8.09±0.18	118.25±17.16	6.51±1.09	3.75±1.49	11.42±4.13	0.54±0.27	0.068±0.014
Naihati	PRM	31.75±1.70	8.23±0.33	125.5±15.67	7.8±2.00	2.16±1.48	6.68±1.74	1.15±0.091	0.063±.026
(G2)	MON	31.12±0.62	8.01±0.05	106.0±37.31	6.2±0.59	4.62±0.84	7.47±2.14	1.05±0.521	0.046±.016
	POM	25.0±2.44	7.85±0.23	113.16±23.62	5.4±1.60	4.88±0.59	15.21±4.14	0.55±0.171	0.060±.011
Dakshineswar	PRM	33.81± 1.13	7.72± 0.29	122.45 ± 9.82	7.04± 0.62	1.22± 0.23	12.82 ±2.64	0.58 ± 0.12	0.12± 0.04
(G3)	MON	30.29± 2.14	7.82±0.38	112.75±8.36	6.15± 0.47	1.65 ±0.23	16.96 ±2.63	1.075±0.23	0.14 ±0.09
	POM	23.61±0.83	7.90±0.37	118.4± 6.63	6.39± 0.61	1.27± 0.15	12.24±2.09	1.25± 0.24	0.145± 0.091
Garden Reach	PRM	34.63 ±1.21	7.77± 0.26	121.61± 8.72	6.32 ± 0.66	1.28± 0.11	11.84±2.27	0.96 ±0.11	0.14± 0.06
(G4)	MON	30.34 ±1.58	7.67 ±0.47	105.49 ± 8.36	6.71 ± 0.28	1.43 ±0.23	15.31±2.14	1.39 ±0.31	0.10±0.03
	POM	18.40±1.03	7.7±0.40	112.48± 9.55	6.92± 0.38	1.26± 0.22	9.15 ±1.13	1.35± 0.15	0.11 ±0.03
Gangasagar	PRM	31.625±5.11	7.68±0.26	574.2±69.78	6.93±0.60	2.45±0.81	31.73±13.02	0.458±0.14	0.132±0.092
(G5)	MON	27.87±1.06	7.57±0.17	450.68±78.91	6.11±0.66	2.86±0.85	21.48±8.44	1.140±0.21	0.06±0.032
	POM	22.8±3.83	7.49±0.34	358.45±96.70	5.93±1.07	2.81±0.56	13.57±2.42	1.365±0.76	0.205±0.168

RESULT AND DISCUSSION

Table 1 & 2 present the excerpt of results of analysis of different limnochemical of River Hooghly at different sampling stations at different seasons. One way ANOVA result revealed that significant seasonal variation ($F=76.52$, $P<0.05$) in temperature was evident in this river. But temperature difference was not significant among sampling stations. Observed water temperature range (18.04 to 34.63) falls within the rage for inland

water in tropics (Imoobe and Koye, 2011). The range of pH in the studied stretch of the river showed sub-alkaline in nature (7.22 to 8.29) that was within the permissible limit as prescribed by WHO (2011). Marked variation in pH was observed among the studied sampling stations as well as between the seasons.

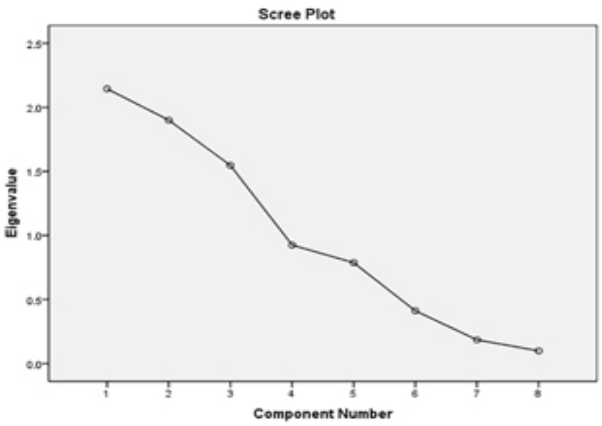
Actually pH level is controlled by various factors including human made waste (Omer, 2010). Water quality can be affected by pH as it can influence the alkalinity, solubility and hardness (Osibanjo et al., 2011). Similar temperature range as the recent study was also reported by Mitra et al., (2018) in same river in the same stretch. The minimal variation in temperature among sampling stations might be attributed to absence of microclimatic variation. Mitra et al., (2018) pointed out the impact of south west monsoon behind such temporal variation in temperature.

Table 3. Rotated component matrix

	Component		
	1	2	3
COD	.837	.385	-.195
Hardness	.773	.495	-.108
pH	-.698	.458	-.120
DO	-.447	.803	.059
BOD	.050	-.710	-.624
Total Phosphorus	.376	-.029	.724
Total Nitrogen	-.110	-.301	.651
WT	-.053	.239	-.378

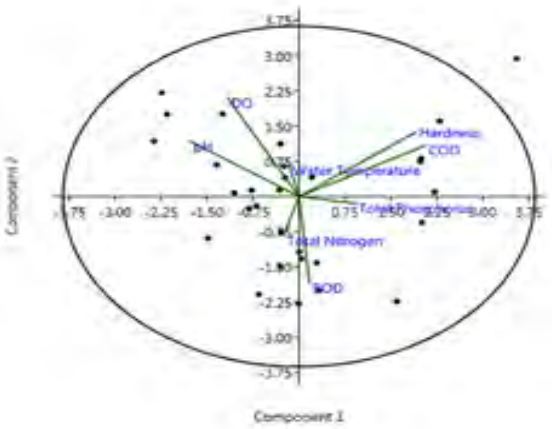
Extraction Method: Principal Component Analysis.
a. 3 components extracted.

Figure 3: Scree plot of PCA sowing all components



From the result it was apparent that the total Hardness level spanned a wide range in the river (86.02 mg/l to 574.2 mg/l). High calcium and magnesium content is responsible for elevated harness level. Hardness values significantly differ among sampling stations as observed in the one way ANOVA results. G5 Sampling station had experienced considerably higher Hardness level all throughout the study period. This might be due to its close proximity with sea. Increased DO values ranging from 4.8 mg/l to 9.3 mg/l is helpful for avoiding the condition of hypoxia threshold (Satpathy et al., 2013; Soo et al., 2016). Our finding is in close conformity with the State environment report West Bengal (2016). Though relatively lower DO level was evident from downstream sampling stations. This range of DO in the lower stretch of River Ganga as observed in our study is very similar to the findings of Mitra et al., (2018) and Dutta et al., (2020); but Nath et al., (2017) reported further lower DO level in this stretch. Significant fluctuations in DO level were evident between seasons. Temporal variation in Phyto-planktonic assemblage, differential organic pollution load might be liable for such differences.

Figure 4: PCA biplot ordination for limnochemical parameters of Hooghly river (factor plane1x2)



High average BOD levels at several points in the Hooghly River like G3 and G4 were observed from CPCB (2013) report and also from recent database of West Bengal Pollution Control Board (WBPCB - www.wbpcb.gov.in). Our finding finds similarity with this report. Higher BOD values at G4 Sampling station can be due to high influx of untreated municipal sewage through Adiganga Chanel, which discharges its content just upstream to G4 sampling site. When raw sewage (both domestic and municipal) is mixed directly with industrial waste water, it exerts a negative impact on self-cleaning activity of the river and the organic pollutants from domestic and municipal sewages diluted quite faster than the industrial inorganic pollutants (Bhaskar et al., 2020). Possibly due to spatial difference in organic pollution load, COD level significantly varied across sampling stations. Total Nitrogen and Total Phosphorus level varied across sampling stations. Phosphate addition can be from industrial effluent, soap and detergents used during bathing and laundry activities. Excessive use of chemical fertilizers in the agricultural lands in this river basin might be responsible for addition of nitrogenous content in the river.

Liu et al., (2003) classified factor loading into 3 classes, these are- strong (>0.75), moderate (0.50-0.75) and weak (0.30-0.50). Component 1 was positively loaded with COD and Total Hardness and negative loadings of DO and pH. Component 2 was dominated by negative loadings of BOD whereas positively loaded with DO. Component 3 is positively loaded with Total Phosphorus, Total Nitrogen and negatively loaded with BOD. This moderate to high loadings of BOD, COD could be attributed to greater array of anthropogenic pollution sources along this river. In component 3 significant positive loadings of Total Phosphorus and Total Nitrogen would be attributed to nutrient enrichment phenomena prevailing at different stations due to surface runoffs and domestic sewage disposal.

According to Rai (2013) numerous industries including textile mills, Paper mills, fertilizer plants etc. are positioned on either sides of Ganga River from the stretch of Uttarakhand to Bengal. Dhara et al., (2015) pointed out the addition of fly ash from different thermal power stations located on the bank of this river and the river water was not suitable even for bathing purpose except the upper stretch (i.e., from Gangotri to Haridwar) (Kamboj and Kamboj, 2019). Dey et al., (2020) also pointed out the degradation of water quality, lack of supportive habitat, over fishing and destructive fishing as the major causes of declination of fish diversity throughout the stretch of river Ganga. Significant results of Barletts test of sphericity indicated that the data was fit for PCA analysis. Application of PCA brought out three (03) factors with >1 eigenvalues. These three (03) factors explained more than 69% variation. In order to identify the number of PCs to discern the nature of underlying data structure the scree plot was applied (Fig: 3). Component matrix and PCA biplot are shown in Table 3 and Fig. 4 respectively.

CONCLUSION

On the basis of different limnochemical parameters investigated, it might be concluded that ecological stresses in the form of raw municipal sewage, industrial wastes and agricultural runoffs have influenced the water quality in all the study areas of the tidal stretch of River Hooghly. Although State and Central Govt. have taken several initiatives to ameliorate the pollution load of river Ganga and some of its tributaries. But the problem is that all the tributaries of river Ganga are not included into these projects. River conservation and management measures in India like most other countries, also suffers from inadequate knowledge of riverine biota. Diminished productivity and less fish diversity in this stretch are responsible for the incurred economic stress to the dependent riverside fishermen. Huge pollution load in the study areas is one of the prime reasons for less production and less diversity. The information coming out of this study will form a base line in adopting future policies and conservation measures for the restoration of ecological health of the concerned riverine stretch. The proper implementation of the research idea can provide a fruitful result in solving the major socio-economic problems of river-side fishers.

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Morphological Traits Associated with Competitiveness in *Oryza sativa*. L: A Case Study

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ABSTRACT

Production of rice suffers a vital force suffering from weed pressure worldwide. Among the weeds, barnyard grass is reported as the most destructive weed species. Synthetic herbicides are preferred method to control weeds. However, continuous application of synthetic herbicides can have a negative impact on the environment, health and result in emergence of herbicide-tolerant weeds. Therefore, another strategy to overcome weed problem is the major concern of scientists. Rice plants with suitable allelopathic trait must be identified which are responsible for secreting secondary metabolites called allelochemicals, and are hopefully very important in controlling weed outbreak.Present work was carried out to evaluate ten rice genotypes based on characteristics related with the competitiveness against weeds. The main plot experiment was conducted in split-plot design with two treatments, weedy and weed free check which was replicated thrice. Morphological parameters such as plant height, tiller number, leaf number and biomass content were measured at flowering stage. Results suggested that rice genotypes reveal variable competitiveness against weeds.

Among the genotypes, highest competitive rate was recorded in Govind and UPR 2962-6-2-1,this could be attributed due to the minimum reduction in plant height (4.0%, 0.6%) .tiller number (4.8%, 9.8%) leaf number(6.4% ,6.6%), and plant biomass content (10%, 5.1%) obtained in rice genotypes respectively at flowering stage. From the study we could assume that morphological parameter can be presuppose to be applied as suitable trait in rice weed interaction for sustainable agriculture. Hence it can be suggested that cultivation of rice varieties having suitable allelopathic potential based on different morphological parameters can be applied which can reduce the heavy stress of herbicides in the rice field and can lead to an increase in the rice productivity in an environment friendly way. It is a biggest challenge for weed scientists to identify suitable trait and incorporate in suitable rice allelopathic cultivar and develop integrated weed management systems that are innovative, effective, economical, and environmentally safe for current and future cropping systems.

KEY WORDS: ALLELOPATHY RICE GENOTYPES, COMPETITIVE ABILITY .

ARTICLE INFORMATION

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INTRODUCTION

Rice is one of the most important food crops of the world with approximately more than half of the world feeding on rice as a staple food crop. But rice production worldwide is severely affected by the weed infestation. The major loss in yield due to weeds infestation is greater than the combined yield losses caused by insect pests and diseases (Asaduzzaman et al. 2010).To control weed outbreak, highest agricultural chemical input is seen in rice in the form of herbicides and weedicides, although these chemicals helps in controlling weeds but, are non-biodegradable and can cause adverse effects after entering into the food chain. Moreover, these chemicals are a major cause of soil and water pollution, thus overall possess a major threat to environment, (Bhadoria 2011; Mohammadi 2013).

So there is a requirement to control weeds without causing a threat to environment. In this respect allelopathy may be an attractive alternative. Growing allelopathic rice to control paddy field weeds is often required to reduce herbicidey dependency and contribute to better approach both for environment and sustainable development of agriculture (Kim, 2011, Kong et al., 2008, Duke, 2010). Many crops including rice have been reported possess allelopathy properties (Dilday et al. 1989; Bhadoria 2011; Bravo et al. 2013; Amb and Ahluwalia 2016, Chung et al 2020).

Rice allelopathy is release of allelochemicals from rice plant itself to suppress weeds growth which is environmentally friendly approach, therefore allelopathy is suggested as a promising approach for biological control of weeds in sustainable agriculture practice (Fang et al. 2013; Khanh et al. 2007).Allelopathic crop varieties can release their own "phytotoxins" as allelochemicals to reduce the growth of weeds, thus permitting ecological weed management in cropping systems (Kong, et al 2011). Rice plants have to face different abiotic and biotic stresses which is responsible for releasing multiple secondary metabolites required to activate defense pathway and protect themselves, (Maruyama et al 2014., Kusano et al 2015).

More than 16,000 rice varieties collected from 99 countries have been screened for their allelopathic capacity, and reported that 4.0% of rice cultivars show weed-suppression of paddy weeds (Khanh, et al 2007).Most allelochemicals are reported as secondary metabolites, which are not essential in primary metabolic processes but play a very important role in defense mechanism ,such as phenolic acids, fatty acids, phenyl alkanoic acids, hydroxamic acids, terpenes, indoles, and the labdane-related diterpenoid momilactones, have been identified as potential rice allelochemicals (Rimando & Duke 2003; Khanh et al. 2007; Kato-Noguchi & Peters 2013). Allelopathic effects can be increased with the increase in temperature and photoperiod conditions, depicting the role of different environmental factors governing the allelopathic traits (Fang et al 2018).

Competitive ability is the joint the contribution of a range of traits that are not only genetically controlled but affected by the growth environment. Genetics and environment together determine the competitive out comein a plant population. These traits can be morphological,or physiological, linked with plant canopy establishment such as early vigour, plant height, growth rate, biomass,leaf area, leaf angle and expansion, tillering capacity, etc. (Olofsdotter et al. 2002). Higher magnitude of leaf area index; higher root growth in-terms dry root weight, length and volume are positively correlated with crop's competitiveness against weeds, (Dass et al 2017).

Rauber (2000) reported that plant height, tiller number and LAI measured at 43 DAS under weedy conditions were positively correlated with weedy yield. In sustainable agriculture, the possibility of incorporating allelopathic character into improved cultivars to enhance competitive ability of rice is worth exploring, (He et al. 2004). Allelopathic varieties can reduce the requirement of commercial herbicides, thus, reducing inputs into agrochemicals (Pervez et al. 2003). Rice allelopathy has attracted great attention since it was demonstrated that some varieties have allelopathic potential against one or more paddy weeds, (Dilday et al. 1989). The current trend is to find a biological solution to minimize the perceived hazardous impacts from herbicides and insecticides in agriculture production. Allelopathy is defined as a beneficial or detrimental effect from a donor plant to the recipient by chemical pathway (Rice, 1984). The harmful impact of allelopathy can be exploited for pest and weed control (Narwal, 1994; Kohli et al., 1998).

Weed control has been an important aspect of their management practices. Although the use of herbicides is a simple and effective method for weed control used worldwide, heavy use of herbicides may cause problems of environmental pollution and soil degradation hampering animal and human health (Chung et al., 1997; Stephenson, 2000). For this reason, various other methods of weed control have been studied. Various studies have employed the exploitation of allelopathic properties in plants which might give promising results (Chung et al., 2003). Dilday et al. (1989, 1991) analyzed 12,000 rice accessions or varieties from the USDA/ARS rice germplasm, many other scientists have documented the allelopathic potential of rice. Different work of allelopathy highly involved the screening of the allelopathic potential of different rice varieties, the exploration of allelochemicals from rice body parts, and the development of new allelopathic varieties (Ahn et al. 2005). Keeping the above points in mind a field experiment was done to evaluate 10 rice genotypes for the allelopathic properties without any herbicide application. The objectives of the present study were to identify plant characteristics which could serve as important selection tool selection criteria for improved wed competitiveness in rice genotypes for high WSA under weedy condition.

MATERIAL AND METHODS

Ten rice genotypes, (*Oryza sativa* L) namely Pant Dhan -16, UPR2916-211, Pant Sankar Dhan -3, UPR-2919-14-1-1, UPR 2962-6-2-1, UPR-2992-17-3-1, UPRI 2005-15, UPR 2805 -14-12, V3R11, Govind were chosen and cultivated under split-plot design at Norman borlogue crop research center, G.B. Pant University of Agriculture and Technology. All the cultivars were maintained under two main plots viz. weedy and weed free. Various morphological and physiological data were recorded. The statistical analysis for all the parameters was done using analysis of variance for split-plot design with means being tested at P = 0.05 using an STPR software designed at the Department of Mathematics, Statistics and Computer Science, CBSH, G. P. Pant University of Agriculture and Technology.

Details of the treatments
Varieties : V1=Pant Dhan 16, V2= UPR 2916-211, V3 = Pant
Sankar Dhan- 3, V4= UPR 2919-14-1-1, V5 = UPR 2962-6-2-1, V6 = UPR 2992-17-3-1, V7 = UPRI
2005-15, V8 = UPR 2805-14-12, V9 = V3R11 and V10 = Govind
Replications 3 Treatments
2 (Weedy, Hand weeding)
Spacing Row to row: 20 cm
Plant to plant: 10 cm
Plot size 3.0 m X 1.8 m

Morphological parameters such as plant height, leaf number, tiller number and biomass production at flowering were recorded.

RESULTS AND DISCUSSION

Various morphological parameters like Plant height, number of leaves, number of tillers, leaf area and dry matter were recorded at the time of flowering and are presented in (Table 1). From the data presented it can be clearly seen that all the parameters recorded showed a decrease in the weedy plots where weeds were allowed to grow with the rice population when compared to the weed free plots.But in some varieties the data for weedy plot is at par or equal to the weed free plots. For plant height, variety V5 has recorded only a mere 0.56% decrease for the weedy plot when compared to the weed free plot, this can be considered at par with the weed free condition. This observation suggests that the plants under weedy condition are growing as luxuriantly as they are growing under weed free condition probably suggesting the allelopathic potential of the rice genotype. Similarly, other parameters like tiller number, leaf number, leaf area and dry matter were also at par for weedy and weed free treatments for the rice variety V5. Also, varieties other than V5 like V9, V10 showed a similar trend like the variety V5 hence these varieties can say to be allelopathic in nature.

In weed free situation the genotype Govind recorded maximum number of tillers at flowering However highest percent reduction was recorded in PD-16.Besides these varieties V6 which showed a marked decrease in all the parameters in weedy condition can be concluded to be the non-allelopathic cultivar. These findings are well supported by the findings of Dilday et al., (1994) Olofsdotter et al., (1995). For example, Dilday et al. (1989) screened approximately 5,000 rice varieties for allelopathy against ducksalad (*Heteranthera limosa* (Sw.) Willd.), of which about 4% demonstrated some allelopathic activity.

The use of allelopathy for weed control has great potential as a biological control method. Despite this, few genetic studies have examined allelopathy (Chang et al 2015) due to the complex challenge of allelopathic interactions in field situations in the presence of natural variability and changing environmental conditions. Parameters of vegetative growth of rice have earlier been correlated with its weed competitiveness. Plant height has often been described as one of the most important factors for total competitive ability of a crop (Gaudet and Keddy, 1988). Plant height of field grown rice can be correlated to the competition of rice plant with the weeds to attain more light. It has been shown that an early increase in the plant height results in lower weed population as it creates pressure on the emerging weed species for light by shading the later, (Khush, 1996; Fisher et al., 1997, 2001).

In the present study, it was found that plant height was higher for the genotypes Govind, UPR 2962-6-2-1 and UPR 2916-211, increase in plant height at flowering suggesting that Govind, UPR 2962-6-2-1 and UPR 2916-211 posed a greater competition on emerging weeds in comparison to other genotypes in early growth stages. Moreover, it was found that Govind and UPR 2962-6-2-1 have a higher yield potential in comparison to other eight genotypes which is consistent with the competitive nature of these genotypes. (Data not shown). Lowest reduction in leaf number, under weedy situation was found in the genotypes UPR 2962-6-2-1 and Govind, hence these genotypes were more competitive while, highest reduction in leaf number was found in UPR 2992-17-3-1 making it least competitive. Tilling ability directly controls the plant's potential to produce a greater number of leaves and a higher leaf area. Production of a greater number of tillers at an early growth phase results in competition imposed on weed seed germination in terms of space and nutrients. Production of high number of tillers under weedy conditions is an important competitive character (Harding and Jalloh, 2011).

Fofana and Rauber, (2000) reported that tiller number measured at 43 DAS under weedy conditions were positively correlated with weedy yield, suggesting that early growth at the vegetative growth stage is essential for high yield under severe weed competition. Also, a high leaf number means, more light absorption, high photosynthesis and consequently a higher yield. In the present investigation, lowest per cent reduction in tiller number and leaf number was found in the genotypes UPR 2962-6-2-1 and Govind. Whereas, PD 16 and

UPR 2992-17-3-1 showed highest per cent reduction in leaf number under weedy situation showing their non-competitive character.

In a similar study by (Saito et al., 2010) reported tillering ability is a key characteristic for WSA under specific growing environments. Total plant biomass is another important characteristic defining the yield potential and weed suppressive ability of the rice plants. Saito et al. (2010) suggested that accumulation of high biomass at early growth stages is a good indicator of competitive rice genotypes. Also (Zhao et al. 2006) reported the role of plant dry matter maintenance under weedy conditions to be an important character for the selection of weed competitive rice cultivars. The shoot extracts of two similar competitive rice genotypes, UPR-2962-6-2-1 and Govind, decreased *E. colona* seed germination, via the release of different phenolic acid compound reported from the plant, (Patni et al. 2019).

Table 1. Morphological parameters recorded at the time of flowering.

		Plant Height		Tiller No.		Leaf No.		Leaf Area		Dry matter	
		Weed free	Weedy	Weed free	Weedy	Weed free	Weedy	Weed free	Weedy	Weed free	Weedy
PD-16	V1	100.8	91.3 (9.4)	12.6	9.7 (23)	32.3	29.9 (7.6)	1572.3	1442.3 (8.3)	14.63	13.63 (6.8)
UPR2916-211	V2	93.3	84.8 (9.1)	8.3	8.0 (4.0)	29.1	29.2 (9.3)	1696.7	1505.4 (11.3)	17.10	15.53 (9.2)
PSD-3	V3	103.8	93.2 (10.3)	12.3	9.8 (20)	34.6	34.7 (13)	1784.3	1574.3 (11.8)	15.90	13.67 (14.0)
UPR-2919-14-1-1	V4	92.8	84.0 (9.5)	10.8	10.3 (4.6)	39	39.0 (13)	1900.7	1669.7 (12.2)	19.37	17.43 (10.0)
UPR-2962-5-2-1	V5	88.8	88.3 (0.6)	8.5	7.7 (9.8)	32.3	39.0 (8.6)	1642.6	1587.3 (3.4)	18.13	17.20 (5.1)
UPR-2992-17-3-1	V6	94	91.3 (9.1)	10.5	10.5 (16)	35.1	35.2 (29)	1380	823.7 (40.3)	18.50	16.30 (11.9)
UPRI 2005-15	V7	101.5	84.7 (16.6)	9.6	8.2 (15)	36	33.3 (9.9)	2002.3	1834.47 (8.4)	13.77	10.63 (22.4)
UPR 2805-14-12	V8	77.5	80.3 (6.2)	9.2	7.8 (14)	35.6	31.0 (13)	1497.7	956 (36.2)	7.77	6.93 (10.8)
V3R11	V9	89.8	82.2 (8.5)	12.3	10.5 (14)	46.8	46.8 (4.7)	1131	1056.24 (6.6)	16.97	15.33 (9.7)
Govind	V10	82.5	79.2 (4.0)	14	13.3 (4.8)	36.3	36.3 (6.4)	949.3	902.23 (5.0)	17.40	15.57 (10.6)
SEm1		0.633		0.184		4.555		13.910		0.529	
SEm2		1.577		0.436		1.669		15.388		0.855	
CV-a		3.873		9.934		15.719		5.350		19.301	
CV-b		4.316		10.526		10.770		4.367		13.942	

(Values in parenthesis depict per cent reduction under weedy situation in comparison to weed free situation)

In the present investigation, the genotypes UPR 2962-6-2-1 and Govind maintained highest plant biomass under weedy condition. Overall, least per cent reduction in plant biomass was recorded for the genotypes Govind and UPR 2962-6-2-1. Similar results were obtained by Saito et al. (2010) who reported that suitable characteristics like shoot dry matter may have great potential for developing high-yielding genotypes under a wide range of weed infestation levels. Different advances in molecular technologies, such as the development of high-density DNA markers, DNA chips, and next-generation sequencing (NGS), have facilitated the identification and characterization of many genes associated with quantitative traits (Chung et al 2020).

CONCLUSION

In the present investigation, an attempt was made to evaluate the competitive ability of ten rice genotypes in terms of their growth physiology at the time of flowering against weeds. Based on the analysis of the data it can be concluded that from all the ten rice varieties under study UPR-2962-5-2-1 and Govind are the allelopathic rice varieties and the rice variety UPR-2992-17-3-1 is regarded as non-allelopathic rice variety. From the study we could understand that morphological parameter can be presuppose to be applied as suitable trait in rice weed interaction for sustainable agriculture. Hence it can be suggested that cultivation of rice varieties having suitable allelopathic potential after assessing the morphological features can substantially be implied which can reduce the heavy burden of herbicides in the rice field and can lead to an increase in the rice productivity in an environment friendly way.

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Influence of High Hydrostatic Pressure on Vitamin C, Beta-carotene Retention, Residual Polyphenol Oxidase and Peroxidase Activity, Antioxidant Capacity and Overall Acceptance of Mango (*Mangifera indica*) Juice

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ABSTRACT

High hydrostatic pressure (HHP) has been considered as one of the most innovative technologies widely applied in food processing by its superior advantages to other conventional processing methods. HHP destroys vegetative cells and inactivates enzymes with an insignificant modification in the organoleptic attributes. Mango (*Mangifera indica* L.) juice is a healthy food drink highly appreciated by its sweet-sour taste and high nutritional components. It's commonly treated by thermal treatment to inactivate microbial and enzyme activity to achieve a long stability in storage and distribution. However, heat seriously affected to sensitive phytochemical components as well as organoleptic properties. Mango juice was treated with hydrostatic pressure under different conditions (150/30, 200/25, 250/20, 300/15, 350/10, MPa/min) to evaluate vitamin C (mg/100g), beta-caroten (µg/g) retention; residual polyphenol oxidase and peroxidase activity (%); free radical scavenging activity (% DPPH), ferric reducing antioxidant power (FRAP, µg/mL); overall acceptance of mango juice. Results showed that vitamin C, beta-caroten, free radical scavenging activity (% DPPH), ferric reducing antioxidant power (FRAP, µg/mL) were highly maintained at 250 MPa in 20 minutes while the residual polyphenol oxidase and peroxidase activity (%) were kept in the highest level. Under the hydrostatic pressure treatment, mango juice also had good overall acceptance. Therefore HPP treatment at 250 MPa/20 min would be ideal in making of mango juice.

KEY WORDS: BETA-CAROTEN, DPPH, FRAP, HYDROSTATIC PRESSURE, MANGO JUICE, PEROXIDASE, POLYPHENOLOXIDASE.

INTRODUCTION

Thermal treatment is a conventional method to inactivate microbial organisms and enzymes in fruit juices; however, heating also causes negative impacts on vitamin C, speeds up decomposition of bioactive constituents, and decreases physicochemical attributes as well as sensory, functional and nutritional values, (Suh et al. 2004; Ndiaye et al. 2009; Patras et al. 2010; Zhang et al., 2016). High hydrostatic pressure (HHP) is one of non-thermal innovative emerging methods not only satisfying the Pasteurization but also significantly maintaining phytochemical components inside fruit juice by the lower processing temperature (Allenda et al. 2006; Rawson and Patras 2011).

Different literatures in the recent past have mentioned that HHP is effective in inactivating microorganisms as

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well as retention of physicochemical characteristics of strawberry juice (Ferrar et al. 2011), pomegranate juice (Varela-Santos et al., 2012), blueberry juice (Barba et al. 2012), apricot, peach, and pear (Anthoula et al., 2014), strawberry juice (Xiamin et al., 2014), beetroot (Paciulli et al., 2016), carrot juice (Zhang et al., 2016), jabuticaba juice (Kim et al., 2017), apple juice (Nayak et al., 2017), grape juice (Chang et al., 2017), mulberry juice (Engmann et al., 2020).

Moreover, high hydrostatic pressure also enhanced antioxidant activity, polyphenol content, flavor, taste and overall acceptability of these juices in comparison to non-pressurized or thermally processed samples. HHP could accelerate the extractability of bioactive elements from food matrix by causing microstructural modification in plant tissues, thus favoring the release of these components (Vázquez-Gutiérrez et al., 2011; Vázquez-Gutiérrez et al., 2013; Xi and Luo, 2016). HPP causes physical damage to the structures of food products, it can also be used as a synergistic extraction technology to enhance the extraction efficiency of functional components, thereby reducing extraction time (Hsiao-Wen et al., 2020). HHP technology offers an effective and safe method of modifying protein structure, enzyme inactivation, and formation of chemical constituents (Gezai et al., 2019).

Mango (*Mangifera indica* L.) has favourable nutrient properties as a source of phenolics, carotenoid, vitamin C, excellent flavour, aroma and colour. It is rich in bioactive molecules protecting human cells from the detrimental effect of free radicals. This fruit is rich in antioxidants potentially reducing the risk of cardiac disease, anti-diabetic, anticancer, anti-inflammatory and antiviral activities (Abbasi et al., 2011; Kalpn et al., 2016; Masud et al., 2016). Because of high perishability, mango fruit becomes rotten quickly and preservation is very essential to make it available for a long stability (Sajeda et al., 2018). Objective of our study focused on the vitamin C (mg/100g), beta-carotene (µg/g) retention; residual polyphenol oxidase and peroxidase activity (%); free radical scavenging activity (% DPPH), ferric reducing antioxidant power (FRAP, µg/mL); overall acceptance of mango juice under high hydrostatic pressure under different conditions (150/30, 200/25, 250/20, 300/15, 350/10, MPa/min).

MATERIAL AND METHODS

Material: Fully ripen raw mango fruits were collected from Soc Trang province, Vietnam. After washing thoroughly with clean water, the fruits were peeled by sharp knife. They were cut into small pieces and then pressed by a screw extractor. The juice was obtained from the filter. The juice was centrifuged at 2000g for 10 min to remove fine solid particles.

Method: Mango juice was filled into polyethylene terephthalate bottles with screw-cup closures. These bottles were placed a hydrostatic pressurization unit. Different conditions (150/30, 200/25, 250/20, 300/15, 350/10, MPa/min) were examined to evaluate vitamin C, beta-carotene retention; residual polyphenol oxidase and peroxidase activity; (%); free radical scavenging activity (% DPPH), ferric reducing antioxidant power (FRAP, µg/mL); overall acceptance of mango juice.

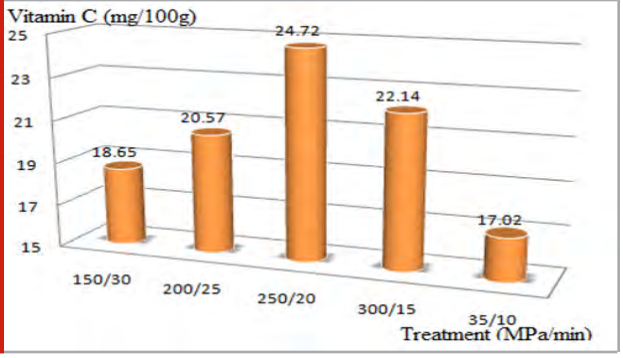
Physicochemical and sensory analysis: Vitamin C (mg/100g) was determined by using 2,6 dichlorophenolindophenol titration as described in the AOAC (2015). Beta-carotene content (µg/g) was analyzed by using high performance liquid chromatography (Nauman et al., 2007). Residual polyphenol oxidase (%) and peroxidase (%) activities were assayed using UV spectrophotometry method proposed by Engmann et al. (2020). DPPH (%) was conducted according to Bakar et al. (2015). FRAP (µg/mL) assay was performed by procedure of Benzie and Strain (1996). Overall acceptance of mango juice was evaluated by a group of panelists using 9 point-Hedonic scale.

Statistical analysis: The experiments were run in triplicate with three different lots of samples. The data were presented as mean ± standard deviation. Statistical analysis was performed by the Statgraphics Centurion version XVI.

RESULTS AND DISCUSSION

HPP application resulted in an instantaneous and uniform transmission of the pressure throughout the product independent of the product size and geometry (Ramirez-Suarez and Morrissey, 2006). As HPP only influenced non-covalent bonds, such as hydrogen bonds, ionic bonds, and hydrophobic bonds, it induced changes in the physicochemical properties and functional activities of biomacromolecules in food products, and even resulted in protein denaturation, enzyme deactivation, and microbe inactivation. In contrast, low molecular weight compounds, such as flavor substances, natural nutrients, and aromatic components, were not affected by HPP (Martinez-Monteagudo and Saldana, 2014).

Figure 1: Effect of HPP treatment (MPa/min) on vitamin C (mg/100g) of mango juice



In our research, bottles of mango juice was treated by HPP under different parameters (150/30, 200/25, 250/20, 300/15, 350/10, MPa/min). Results revealed that vitamin C (mg/100g), beta-carotene (µg/g), free radical scavenging activity (% DPPH), ferric

reducing antioxidant power (FRAP, µg/mL) were highly maintained at 250 MPa in 20 minutes while the residual polyphenol oxidase and peroxidase activity (%) were kept in the highest level. Under the hydrostatic pressure treatment, mango juice also had good overall acceptance (figure 1-7). Yen and Lin (1996) reported that 11.32% of ascorbic acid in strawberry coulis decreased after HHP treatment at 400 MPa/20°C/30 min.

Figure 2: Effect of HPP treatment (MPa/min) on beta-carotene (µg/g) of mango juice

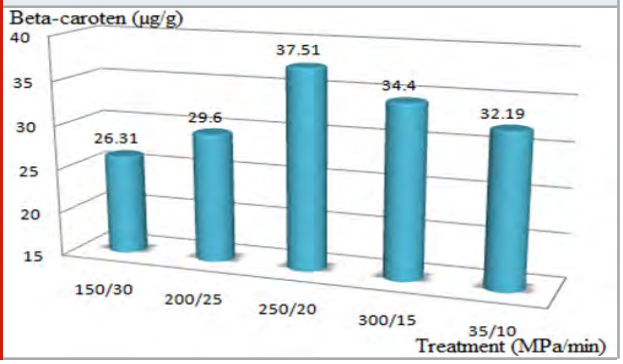


Figure 3: Effect of HPP treatment (MPa/min) on DPPH (%)of mango juice

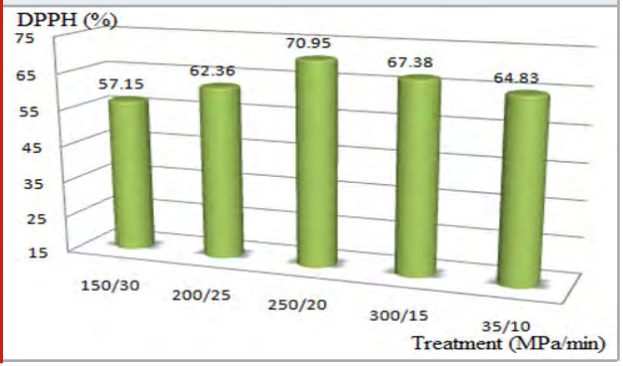
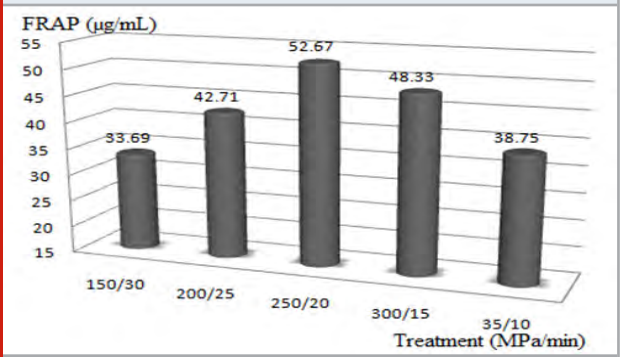


Figure 4: Effect of HPP treatment (MPa/min) on FRAP (µg/mL) of mango juice



HHP can improve cell permeability due to its aptitude to deprotonate charged groups and disrupt salt bridges and hydrophobic bonds in cell membranes; therefore, the extraction of polyphenols from pulp particles is more accessible, (Xiamin et al., 2014). Antioxidant activity of strawberry and blackberry purees was reduced by 25% by thermal processing but there was only 5% reduction treated by HPP with better antioxidants and ascorbic acid retention, (Gezai, 2019).

Figure 5: Effect of HPP treatment (MPa/min) on residual PPO (%) of mango juice

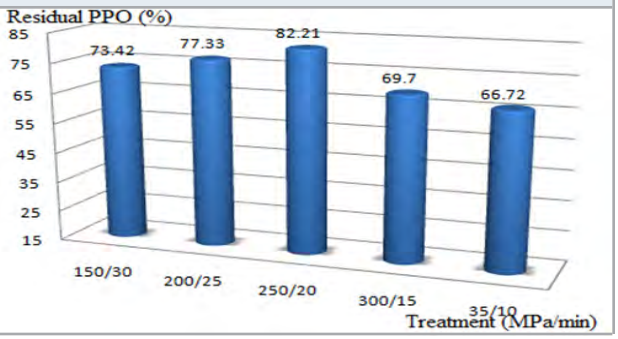


Figure 6: Effect of HPP treatment (MPa/min) on residual PO (%) of mango juice

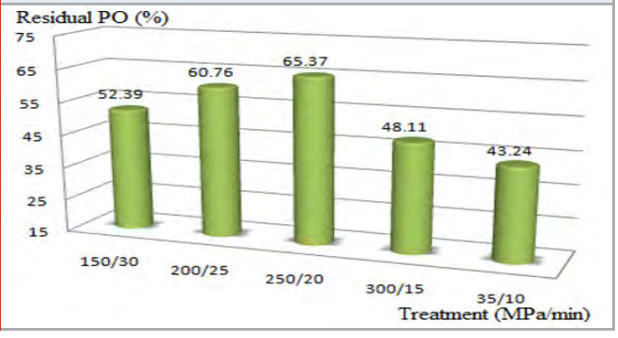
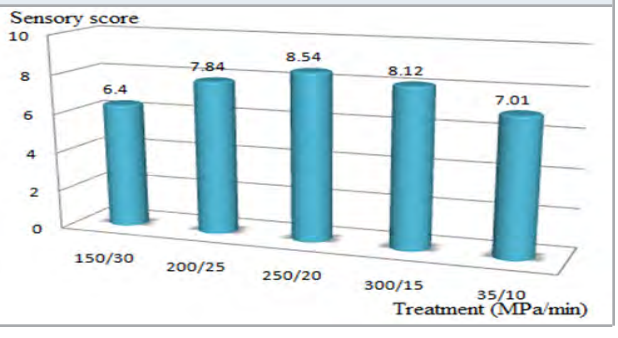


Figure 7: Effect of HPP treatment (MPa/min) on sensory score of mango juice



Aaby et al. (2018) proved that HPP did not result in significantly different vitamin C contents in strawberry juice. Andrés et al. (2016) showed that the total carotenoid content of soy-smoothies increased after HPP (650 MPa/3 min). Ali et al. (2019) demonstrated that HPP resulted in a substantial retention of vitamin C content of wheatgrass juice. Some studies reported that HHP could not effectively inactivate polyphenol oxidase, peroxidase in fruits and vegetables (Goodner et al., 1999; Corredig et al., 2002; Baron et al., 2006; Dalmadi et al., 2006). The antioxidant capacity in mango juice after HHP treatment was evaluated using•DPPH and FRAP methods. Phenolic compounds were responsible for

antioxidant capacities in fruits, and the fruits with higher phenolic contents generally showed stronger antioxidant capacities. Sánchez-Moreno et al. (2006) indicated that total scavenging activity (DPPH) in aqueous and organic fractions of tomato puree was unaffected by HHP treatment at 400 MPa/15 min/25°C.

Chaikham and Prangthip (2015) found that DPPH radical inhibition (%) and FRAP value (mMFeSO4/g) revealed a higher antioxidant capacity in pressure-treated longan flower-honey. González-Cebrino et al. (2016) proved that HHP influenced the volatile constituents of red plum purée. Hartyáni et al. (2011) and Ferrari et al. (2010) demonstrated that the aroma of HHP-treated citrus and pomegranate juices, could be significantly different from that of the fresh juice. Oey et al. (2008) suggested that HHP resulted in slightly modified organoleptic properties. Engmann et al. (2020) demonstrated that hydrostatic pressure treatment at 200 MPa in 10 min showed a better inactivation of enzyme activity, higher conservation of anthocyanin of the mulberry juice.

CONCLUSION

High hydrostatic pressure is an innovative processing strategy that includes subjecting fruit juice to high isostatic pressures, causing microbial and enzyme inactivation to ensure improvement of food safety and stability of perishable components, while maintaining nutritional, functional and organoleptic properties. Consumers are demanding minimally processed and fresh food products, the application of non-thermal techniques like HHP is gaining popularity.

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Conflict of Interest: The authors declared that present study was performed in absence of any conflict of interest.

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Attachment, Proliferation and Differentiation of Mesenchymal Stem Cells on Implant Coated with Chitosan

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ABSTRACT

Osteogenesis is characterized by a serial of events involving cells attachment, proliferation, and differentiation. However, chitosan applications in osteogenesis mechanisms have remained limited. This study intends to examine chitosan's effect with different degrees of deacetylation (DDA) as a coating material for the Resorbable Blast Textured (RBT) implant surface. 63 Resorbable Blast Textured discs were coated either with 80 or 95 DDA. These discs were categorized into three groups i.e., RBT 80, RBT 95, and RBT control (without coating). After their separate applications, Cell viability, morphology, and bone formation were studied at 7 and 14 days. All samples showed biocompatibility and allowed cell attachment. However, areas with high cellular density were found in abundance around surfaces coated with chitosan in comparison with the control. At day 14, test groups coated with chitosan, especially 95 DDA, showed significant mineralization process and growth of nodule-like structures of hMSCs on the surfaces. No significant differences were found in cell viability except for RBT 80, which was lower in comparison to other groups. RBT 95 showed a significant increase in all osteoblast markers in comparison with RBT 80 and control. Chitosan material was confirmed as a right candidate for implant coated with Resorbable Blast Textured surface.

KEY WORDS: BONE CELL; CHITOSAN FILM; DEGREE OF DE-ACETYLATION, IMPLANT COATING.

INTRODUCTION

It is well recognized that functional allogenic tissue development necessitates the coordination of cell adhesion, growth, differentiation, and organization into a particular tissue architecture (Rogina et al., 2017). Similarly, the development of the stem cell microenvironment has become critical for regenerative medicine (Bardelli and Moccetti, 2017). Studies show mesenchymal stem cells (MSCs), which are isolated from the adult bone marrow, possess self-renewing capability which able to differentiate into various cell phenotypes, showing their potential beneficial use for bone tissue regeneration. Concerning the implant therapies, various studies show functional as well as biological advantages for the patients in contrast to the conventional method, providing benefits in the long-run. This is evident from the ten years long study with survival and success rate of 95 percent (Buser et al., 2012; Fischer and Stenberg, 2012, Li et al., 2016, Mora et al., 2017).

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Mainly, the success of the implantation depends on osseointegration, which constitutes bone modeling and remodeling processes (Li et al., 2018). Optimal osseointegration depends on the material implant characteristics, implant loading, and surgical techniques as well as the quality, distribution, and amount of bone present at the implant insertion site (Guglielmotti et al., 2019, Chen et al., 2019). Accelerating this process is a modern trend in implant dentistry, which relevance increases when treating medically compromised patients such as diabetic and osteoporotic patients (Naujokat et al., 2016, Vohra et al., 2014). To minimize the treatment time, the implant surface coating of the biomimetic materials or agents is used. In this regard, increased use of chitosan (CH), a natural biocompatible polysaccharide derived from the crustacean shells, has been observed (Elieh-Ali-Komi and Hamblin, 2016). Chitosan comprises various polymers with a varying differences in molecular weight, degree of deacetylation (DDA), and viscosity (Kumaran, 2020).

Su et al., (2017) have reported an increasing level of resemblance between the bone and cartilages extracellular matrix and CH components and chemical structure. Arunkumar et al. (2017) showed that CH components also denotes effective osteoconductivity, which help improve the vitro as well as vivo tissue generation (Arunkumar et al., 2017). Other biological properties have been indicated by previous searches on CH include antitumor, antioxidant, and antimicrobial characteristics (Pippi et al., 2017, Costa et al., 2014, Cheung et al., 2015). However, these properties are likely to be affected by DDA, which refers to the deacetylated units' molar fraction or deacetylation percentage and the molar weight of CH (Jiang et al., 2017). Most studies highlight the impact of chemical and physical properties of DDA on CH as a coating material (Cheung et al., 2015; Bumgardner et al., 2007). For instance, one study revealed that different DDA of chitosan as implant coating did not affect the cell growth nor the degradation rate. However, the tensile bond strength was lower, with 81.7% DDA (Yuan et al., 2008). However, Limited studies have focused on the effect of DDA on chitosan coating potential.

Although chitosan has been approved to be an excellent coating material and was investigated using different in vitro/vivo models(Govindharajulu et al., 2017, Husain et al., 2017), previous studies were focused on coating a commercially pure titanium surface, and no study used different surfaces to test the coating potential. Therefore, the aim of this investigation was to determine the morphology, proliferation, and pattern of attachment of hMSC-TERT 20 on chitosan with two degrees of deacetylation (DDA) as a coating material for Resorbable Blast Textured implant surface (RBT).

MATERIAL AND METHODS

Materials: The powdered form of chitosan of about 200 kDa molecular mass and 500 mPas viscosity with two different DDA 80 and 95 were used (Heppe Medical Chitosan GmbH, Germany). 63 Textured implant surface (RBT) discs with a diameter of 10 mm (Biohorizon company) were utilized. The discs were divided into three groups with 21 discs for each: RBT 80, RBT 95, and RBT control.

Coating Procedure: The chemical bond between the CH material and the disc surface was created through a silanization reaction adapted from Bumgardner et als methodology with some modification (Bumgardner et al., 2003b). Briefly, the disc surfaces were suspended in water/ethanol solution (5:95 vol %) acidifying to 4.5 pH and 10 M acetic acid. Following it, 2 vol% silane-coupling agent was added for ten minutes at room temperature, whereas pH level was sustained at 4.5 to 5.5. The non-adhered silane was removed by rinsing the disc with ethanol and was cured at 110±C. Then, the implanted disc was suspended overnight in glutaraldehyde solution (2 vol %), with a pH of 4.3 at room temperature. After this, a solution of CH (2 wt. %) was prepared with acetic acid (0.2%) at room temperature.

For eliminating the undissolved particles, the CH was centrifuged before casting. Later, the chitosan was kept at 4^{0}C overnight. Next, the disc was cast with CH solution of 1ml at room temperature. Water was allowed to evaporate over 5-7 days. After coating and before seeding, 1disc from each group was studied under the scanning electron microscopy (SEM). Implant coated with chitosan were sterilized using ultraviolet (UVUV) light for an hour followed by ethanol soaking (70%) for two hours, and then it was washed using Phosphate Buffer Saline (PBS) twice (Govindharajulu et al., 2017, Abuelreich et al., 2017).

Cell Culture: Both coated and non-coated discs were placed in individual wells. For human bone marrow-derived mesenchymal stem cells (hBM-MSCs), hMSC-TERT 20 passage 54 were used. The cell growth occurred in a media which consist of DMEM (ATCC, Manassa, VA, USA) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin-Streptomycin solution, 100X (10,000 Units/ml Penicillin +10,000 µg/ml Streptomycin) and 1% MEM Non-Essential Amino Acids Solution 10mM (100X, Cat No RMNAA-0100X) under standard cell culture conditions (37^{0}C,100% humidity, 95% air, and 5% CO2). Following 80-95% of cells confluence, they were collected, washed, and counted with a hemocytometer. The study used 1x10^6 cells, which were plated with culture medium onto implant discs and incubated for 24h to facilitate adherence to the surface.

After that, the media was changed for each implant disc and the cells were grown and maintained in an osteogenic medium which comprised of 100 nmol/L dexamethasone (Sigma-Aldrich, Cat No D1756-1G), 10 mmol/L Sodium β-glycerophosphate pentahydrate (Loba Chemie Ltd., India, Cat No 05885), 50 g/ml L-Ascorbic Acid, Vitamin C (Winlab Ltd., UK, Cat No107888), and Cholecalciferol, (+)-Vitamin D3 (Cat No C9756-1G). The media was changed every three days.

Cell Viability Assays: AlamarBlue (ABAB) assay was used to evaluate the cell viability (AbD Serotec, Raleigh, NC, USA). For each reaction, 10 % of Alamar blue was diluted into 100 µl of DMEM and incubated in the dark for 60 minutes at 37°C. The samples were read through a microplate reader (Synergy™ 2 Multi-Mode Microplate Reader) at 590 nm. Three readings were recorded for each sample at each time point.

Quantitative Real-Time-Polymerase Chain Reaction (qPCR): RNA isolation was accomplished at days 7 and 14 using recommended BIOFACT HiGene Total RNA Prep Kit (Biofact, Cat No RP101-100, Korea). RNA concentration and protein contamination were measured using a spectrophotometer (Eppendorf-Biospectrometer basic). Samples with ratios between 1.8 and 2.0 purity were used. Amplification and synthesis of Complementary DNA (cDNA) was done from lµg of RNA using a FIREScript RT cDNA synthesis KIT (Solis BioDyne, Cat No 06-15-00200). Using a total reaction volume of 20µl and HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Cat No 08-36-00008), QPCR were completed. For normalization, B-actin was added for all the reactions as a control gene. All qPCR assays were performed and accomplished under the same conditions as triplicate (n=10, duplicate independent experiments). The primers' sense and antisense were designed based on GenBank. The primers used in this experiment are listed in Table (1).

Table 1. Primers Sequence

Sequence Name	Description
OSTEONECTIN F	5' GAG GAA ACC GAA GAG G 3'
OSTEONECTIN R	5' GGG GTG TTG TTC TCA TCC AG 3'
RUNX2 F-RO	5' GTA GAT GGA CCT CGG GAA CC 3'
RUNX2 R-RO	5' GAG GCG GTC AGA GAA CAA AC 3'
OSTEOCALCIN R-RO	5' CTC ACA CAC CTC CCT G 3'
OSTEOCALCIN F-RO	5' GGC AGC GAG GTA GTG AAG AG 3'
ACTB (Beta-actin) R	5' ACATCTGCTGGA AGGTGGACA 3'
ACTB (Beta-actin) F	5' TCAAGATCATTGC TCCTCCTGAG 3'
ALPL F	5' GACGGACCCTC GCCAGTGCT 3'
ALPL R	5' AATCGACGTGGG TGGGAGGGG 3'

Measurement of Osteoblast Differentiation by Alkaline Phosphatase Activity: The alkaline phosphatase activity was measured quantitatively by using the Alkaline Phosphatase Activity Colorimetric Assay kit (BioVision, Cat No K412 -500ASSAY). In brief, after the induction of cells on 7th and 14th day, the culture medium was removed and washed thrice using PBS. The cells were collected from the wells through trypsinization using 25% trypsin and afterward, centrifuged at 7500 rpm for 5 minutes. Next, the harvested cell pellet was treated by the kit while following the company's instruction manual. At 405 nm, the absorbance was measured in the microplate reader.

Cell Morphology: Scanning Electron Microscopy (SEM) was used for the examination of the cell culture at time intervals of 7 and 14 days. Before observation, the PBS was used to wash the implant discs thrice for eliminating all non-adherent cells and fixed in 4% v/v glutaraldehyde at 4°C overnight. Followed by the gradual immersion of the sample in water-ethanol solution. For osteoblast adhesion, Rhodamine-phalloidin staining was used to view the cellular actin filaments. After incubation of cells for 24hs, 4.0% paraformaldehyde was used to fix the samples for 15 minutes. Next, the cells were blocked by using 1.0% BSA for 30 minutes and then kept overnight at RT. The next day, the samples were stained for 30 minutes with 40, 60-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO). The confocal microscope was used to obtain the fluorescence images (a Zeiss LSM510 META confocal system, connected to an inverted Zeiss Axiovert 200 microscope).

Ethical Approval: The study protocols, as outlined by the Kind Saud University Approval of the Ethical Board Committee, were followed. Institutional Review Board (No E-18-2850) as well as Dentistry Research Center (CDRC No. PR 0074) provided ethical consideration. The Molecular and Cell Biology Laboratory provided the hMSC-TERT20 in collaboration with Prince Naif Bin Abdulaziz Health Research Center.

Statistical Analysis: IBM SPSS (Statistical Package for Social Sciences/ SPSS 22; IBM Corp., NEW YORK, NY, USA) was used for the analysis of the results. The normality of the data was assessed by using the Shapiro-Wilk test. The statistical significance between groups were determined by using a two-way analysis of variance (ANOVA) followed by one-way analysis of variance (ANOVA) and the Tuckey post hoc assessment. Mean, and the standard deviation was computed for the measurable data. The significance value was determined at $P \leq 0.05$.

RESULTS AND DISCUSSION

The structural and chemical properties of chitosan make it an excellent alternative to traditional coating materials such as calcium phosphate (van Oirschot et al., 2016). This is consistent with the previous studies which considered chitosan as an osteoinductive material which can act as antimicrobial, accelerate wound healing, and can promote bone formation (Li et al., 2015, Pippi et al., 2017). The objective of this project was to determine the behavior of hMSC on implants with rough topography. To evaluate cell viability and attachment, the Resorbable Blast Textured implant surface was coated with chitosan

with two different DDAs. First of all, the coating architecture was studied under SEM for all groups. At a magnification of 1000, RBT control surface demonstrated multiple irregularities with small flaws, depressions, and spikes (Figure 1).

Figure 1: (a) gross view of Resorbable Blast Textured (RBT) surface without coating, (b) under SEM. (c) RBT surface coated with chitosan 80 DDA, (d) under SEM. (E) RBT surface coated with chitosan 95 DDA, (E) under SEM. All of the samples at a magnification of 1,000

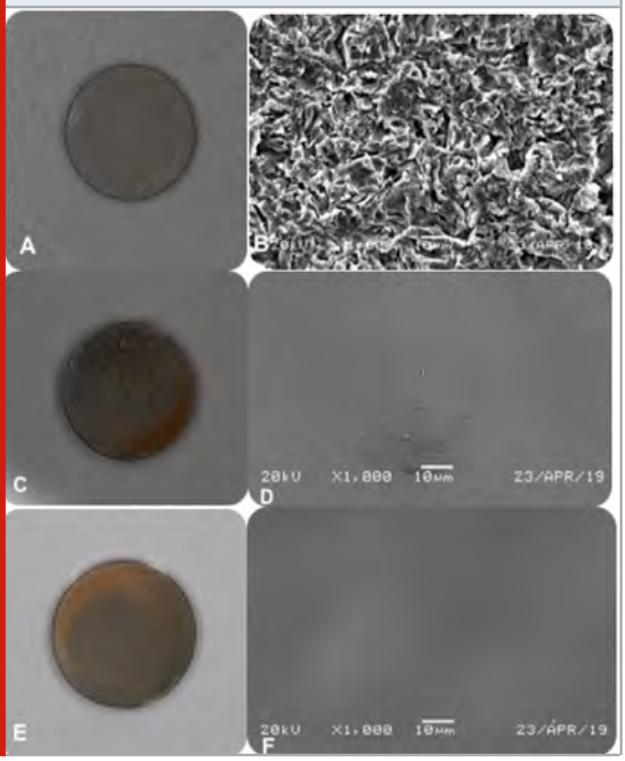
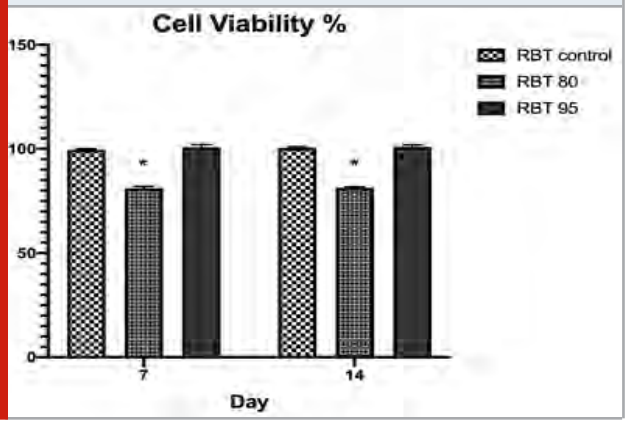


Figure 2: Alamar blue quantification for cell viability performed on cells grown on control or chitosan 80/95 coated groups during osteogenic differentiation for 7 and 14 days. Values were presented as mean ± SDSD of six different groups. *p < 0.05



For chitosan 80 or 95 DDA, all of them showed a similar appearance in which they formed a homogenous and transparent coating, which is free from any cracks. The chitosan molecules chain was attached to the disc surface by their NH2 groups. This attachment occurred through their silane-glutaraldehyde molecules, which account for the yellowish tint on all the coating (Yuan et al., 2008). Studies demonstrated that the silanization method is not only simple and cost-effective but also chemically binds the substrate to the implant surface, which confirmed by mechanical tests (Yuan et al., 2008, Bumgardner et al., 2003b).

The AlamarBlue results showed that no significant difference in cell viability between control and RBT 95 during day 7 and day 14 (Figure 2). However, RBT 80 was significantly lower in cellular viability at both time intervals in comparison with the other groups. This indicates that these surfaces were biocompatible and would not be cytotoxic in vivo, even with time progress. However, even though proliferation was lower in RBT 80, still full confluent cell layer and attachment were obtained at 7 and 14 days. Also, according to the International Organization ISO10993-5, if the cell viability remained >70%, then the material does not have cytotoxicity potentials (10993-5, 2009). One study found that cell attachment and proliferation was increased by chitosan with high DDA membranes which coated with fibronectin (Lieder et al., 2012).

This is different from the study findings of Abuelreich et al., which showed that cells cultured on the Chitosan-Polycaprolactone membrane had more cell attachment and less proliferation than plastic surface control (Abuelreich et al., 2017). Another study showed that keratinocyte was increased in proliferation when the DDA decreases; thus lower DDA will increase the cell adhesion. On the contrary, fibroblast demonstrated a better adhesion than keratinocyte, although they remain alive, they do not proliferate well regardless of the DDA. This kind of cell behavior was related to the high adhesion to the underline surface in which inhibits their growth (Chatelet et al., 2001). After that, the capacity of osteoblast cell growth was studied by using molecular assays.

Coated and non-coated samples were evaluated for the expression of Runx2, ALP, and matrix mineralization. Cells grown on chitosan-coated material have more expression of bone markers in comparison with the control group. Moreover, 95 DDA caused statistically higher bone marker expression than the other groups (Figure 3). Alkaline phosphatase (ALP) expression is the most commonly used marker for bone formation (Choi et al., 2011). Chitosan material influenced ALP activity at 7 and 14 days from cell culture with the highest activity on 95 DDA (Figure 3-4). The quantity of ALP enzyme was significantly lower in RBT control in comparison with the other groups. One study found that after 8 days of incubation, ALP's intracellular activity was higher on chitosan-coated titanium discs compared with the

pure titanium group. However, they used a combination between 85 and 90 DDA (Li et al., 2015).

Figure (3): Osteoblast gene expression at 7 and 14 days from cell culture.

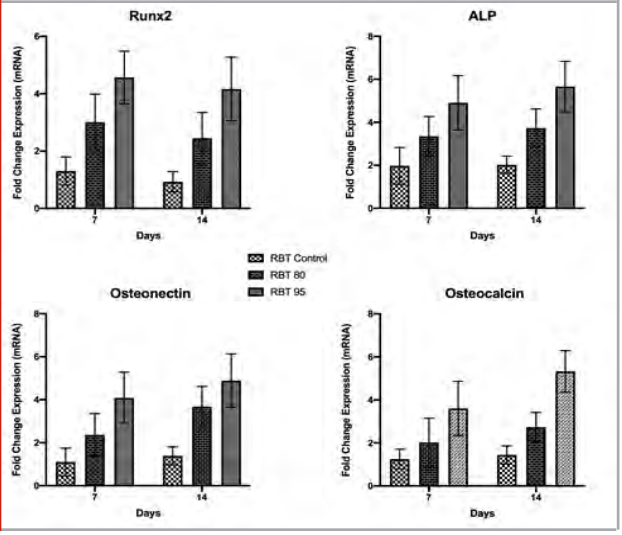
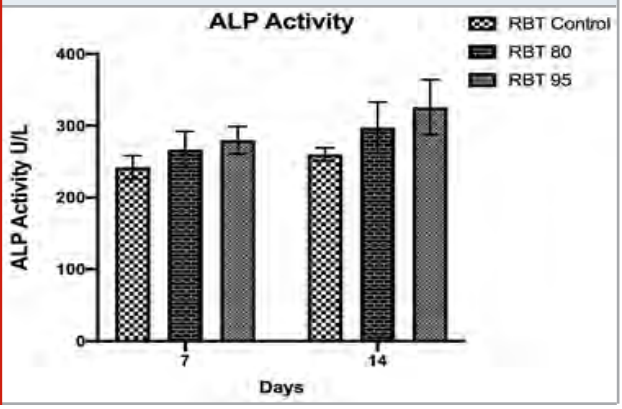


Figure 4: Alkaline phosphatase activity at 7 and 14 days of cell culture



Mineralization process can be detected by measuring the amount of Osteocalcin (OC) and Osteonectin (ON) activity (Ozdemir et al., 2016; Rosset and Bradshaw, 2016). The expression of these two non-collagenous markers increases in the final phases of bone formation by mature osteoblasts. Our data displayed that these genes were expressed more in chitosan-coated samples in comparison with the RBT control, and by following the general trend of bone mineralization, the expression of ON and OC was more at day 14 in comparison with day 7. A study by Mathews et al. examined different densities of chitosan with >87.61 DDA coating for osteoblast differentiation. Chitosan coated plates revealed more gene expression of osteoblast markers, including Runx2, ALP, ON, OC on day 7, 14 and 21 from cell culture in comparison with untreated plates. Moreover, calcium deposition measured by quantification assay was 30% more on chitosan-coated plates by day 14 and 21 from osteogenic induction (Mathews et al., 2011).

Figure 5: (G–L) Scanning electron microscopy (SEM) images. (G) RBT control at day 7 (H) at day 14. (I) RBT 80 at day 7 (J) at day 14. (K) RBT 95 at day 7. (L) At day 14. 2500× magnification.

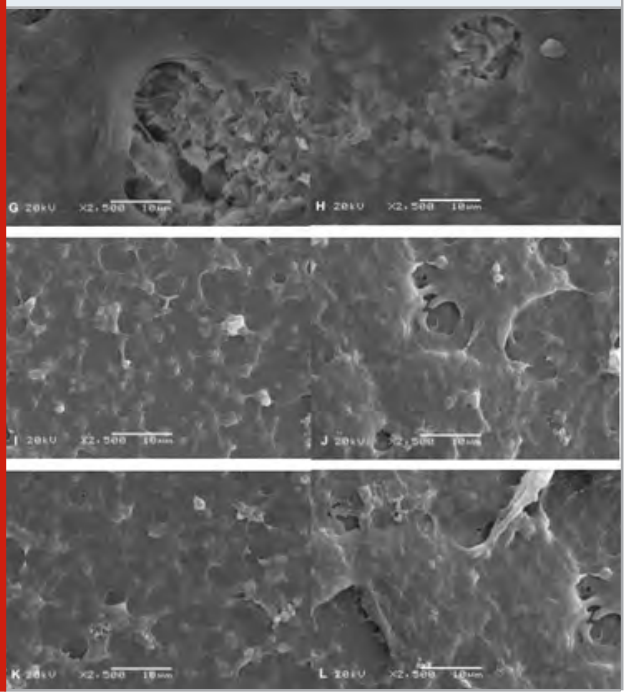
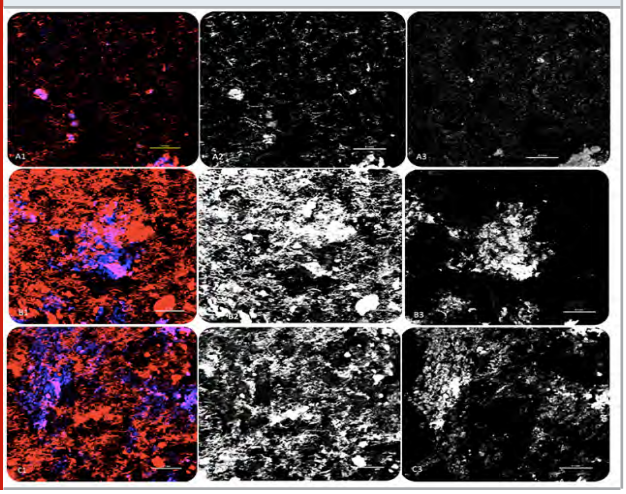


Figure (6): Images of cell morphology and osteoblast adhesion. (A1) for RBT control as all. (A2) with Phalloidin for actin filaments. (A3) nuclei stained with DAPI. (B1) for RBT 80 as all. (B2) with Phalloidin for actin filaments. (B3) nuclei stained with DAPI. (C1) for RBT 95 as all. (C2) with Phalloidin for actin filaments. (C3) nuclei stained with DAPI.



According to the literature, the deacetylation degree in the chitosan is high with an increased number of amino groups, which is better for implant surface bonding. Thereby, an increase in the amino group is likely to increase the positively charged particles, which leads to a high net positive charge on the coated samples. Consequently, the coating will have a higher affinity for

negatively-charged cells and growth factors (Bumgardner et al., 2003a, Hamilton et al., 2007, Prasitsilp et al., 2000, Fakhry et al., 2004). Overall, all surfaces showed good biocompatibility and allowed for cell attachment, proliferation, and differentiation. After 24 h, the confocal results indicated that hMSC could attach and grow on chitosan-coated surfaces as well as control.

For RBT 95 and RBT 80, dense flattened osteoblasts approximating each other were seen on the top of the surface (Figure 6). For SEM results, the samples coated with chitosan showed more layers of dark cell patches overlapping each other while forming bridge-like structure at day 7 (Figure 5). With a later stage, the calcium deposits were clearly visible at the chitosan-coated groups in contrast to the control groups. The nodule-like structures suggested that the cells were committed to developing osteogenic phenotype and osteoblasts differentiation (Abuelreich et al., 2017).

Concerning the different DDAs, the results of the present study showed that 95 DDA coated chitosan is superior in terms of biological responses in comparison with 80 DDA coated chitosan. Research by Foster and his group showed that cell line, which grew on 85 DDA was much better in cell spreading in comparison with 72 DDA (Foster et al., 2015). On the contrary, A recent study investigated the influence of DDA on the biological behavior of the MC3T3-E1 cells and found that a DDA in the range of 87–94% had a less critical effect on cell growth or proliferation in comparison with the source of chitin (shrimp & crabs) (Supernak-Marczewska and Zielinski, 2020). To the best of the researcher's knowledge, this is the first study that utilized chitosan to coat implant surface other than pure titanium, and these data encourage future researchers to do more investigation regarding the mechanical properties of this combination for future implant design.

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(2) Book: Falconer DC (1960) Introduction to Quantitative Genetics. Oliver & Boyd, Edinburgh 165-185.

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