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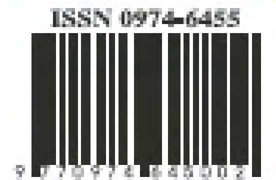

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Characteristics of Peptone from the Mackerel, Scomber japonicus Head by-Product as Bacterial Growth Media

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ABSTRACT

Mackerel (*Scomber japonicus*) by-products such as head part is generated by fish processing industries and it may result in serious environmental problem. One of the best approaches to utilize mackerel by-product is to recovery into fish peptone. Fish peptone can be hydrolyzed by adding acid compounds that mostly categorized into positive list compounds in terms of halal or kosher view point. The aim of this research was to characterize peptone extracted from mackerel head by-product as bacterial growth media. Hydrolysate of mackerel head by-product was treated by Hydrochloric acid (HCl) to extract the peptone. The characteristics of extracted peptone was analyzed by determining chemical composition (total nitrogen, fat, ash, and moisture content), color, solubility, bacterial growth, and biomass properties. The results revealed that the chemical composition of peptone extracted from mackerel head by-producs consist of moisture 5.07%, total nitrogen 11.53%, fat 1.33% and ash 3.78%. The color intensity of mackerel by-product peptone was 87%, it showed lower intensity than commercial peptone (90.71%). The values of pH and solubility were 6.9 and 97.07%, respectively. In the bacterial growth test, the peptone extracted from mackerel by-product product showed faster bacterial growth rate (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thyphi*, and *Aeromonas hydrophila*) and higher biomass production than the commercial peptone. This findings revealed that mackerel head by-product is a potential raw material for production of peptone as media for bacterial growth.

KEY WORDS: *BACTERIAL CULTURE MEDIA, MACKEREL HEAD BY-PRODUCT, PEPTONE, PROTEIN HYDROLYSATE.*

ARTICLE INFORMATION

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INTRODUCTION

Peptone is water-soluble protein, especially rich in amino acid composition that is uncoagulatable by high temperature. It has a wide range of biological and biotechnological applications, such as microbial substrates in supporting growth rate and biomass production. Most of the produced peptone is derived from terrestrial animals like cow and pig and their derivatives. Small amount of peptone also produced from plants and yeasts (Fallah et al., 2015). However, cow peptones facing the risk of outbreaks of diseases, such as bovine spongiform encephalopathy (BSE) or mad cow disease (MCD), while pig peptones are related to the halal and khoser issues in terms of religious beliefs, such as Islam and Judaism. Hence, there is a considerable interest in replacing land animals as raw materials of peptones production, and one of the proposed alternative sources is fish materials. FAO (2018) reported the global fisheries production in 2016 reached 170.0 million tonnes, consists of 80 million tonnes (representing 47%) in aquaculture and 91 million tonnes (representing 53%) in capture. Mackerel is one of the economically important species in the world, accounting for 1.6 million tonnes of total global fisheries production in 2016. Generally, mackerel processed in the form of a fillet and canned fish products. Among them, about 30% of the total mackerel processing industry are by-products, including head, viscera, fins, and scales (Villamil et al., 2017).

Mackerel head by-product can be utilized as potential fish peptone that has more acceptable and marketable than other utilized products. On the other hand, utilizing fish by-product could prevent serious environmental problems (Herpandi et al. 2011). Fish peptone can be hydrolized by adding enzyme and acid compounds. Enzymatic hydrolysis could breakdown peptide bonds of proteins or polypeptides into smaller amino acids, but it has drawbacks, such as slow reaction rate and high cost, as a result, less effective in the large scale production (Khalil, 2012). On the contrary, acid hydrolysis has advantages, including low cost, short hydrolysis period, simple operation and applicable to industrial processes (See et al., 2011). Benites et al. (2011) stated that the selection of acidifying agent is based on three factors such as cost, availability, and bactericidal action. In additon, acid solutions are mostly catagorized into positive list compounds in terms of halal or kosher view points. For microbial growth and biomass production, peptone is an essential source of nitrogen due to the high polypeptides and amino acids. Aspmo et al. (2005) revealed that an important component in microbial growth media is a nitrogen compound. Several studies have been observed that peptone extracted from sole fish by-product was capable of supporting microbial growth rate in the cultures of *Pseudomonas aeruginosa*, *Lactobacillus acidophilus*, and *Saccharomyces cerevisiae.*

Furthemore, recently, Shirahigue et al. (2018) reported peptones are hydrolyzed from tilapia and cobia by-product with different acid solutions (citric, formic, and propionate acid) had a significant growth rate and biomass production in the culture of *Escherichia coli* and *Staphylococcus aureus* compared to commercial peptone. These findings also in accordance with the peptone isolated from Atlantic cod stomach with formic and phosphoric acid reported by Gildberg et al. (2010). As mentioned above, fish peptones provide better microbial growth, as well as higher biomass production due to the high content of soluble protein compounds, particularly nitrogen compounds. In addition, fish peptones are acceptable for all religions and do not related to dengerous diseases. However, few investigations on peptone hydrolyzed from mackerel head by-product with acid-adding process. This reseach aimed to characterize peptone from mackerel (*Scomber japonicus*) head by-product using acid-adding process as bacterial growth media in comparison with commercial peptone.

MATERIALS AND METHODS

Materials: The mackerel heads were obtained from PT. Kelola Mina Laut (Gresik, East Java, Indonesia). The average weight of a mackerel head was 14.5 g. The mackerel heads were put in polyethylene plastic and conditioned at cold temperatures (4°C) during transportation. After the sample arrived at the laboratory, the sample was washed with running water and grounded using a milling machine (Maxindo, MHW-80, Indonesia). Then, sample was put into polyethylene plastic and stored in the freezer at a temperature of -20°C for up to 1 week. *Escherichia coli, Streptococcus aureus*, Salmonella thyphi. and *Aeromonas hydrophila* strains used in this research was obtained from InaCC LIPI, Indonesia. All chemicals and reagents used were of analytical grade. Preparation of mackerel head by-product hydrolysate: Hydrolysate of mackerel head by-

product according to the method carried out by Khalil (2012) with slight modifications. Samples were weighted 900 g by digital weight machine, and then, added 10% of distilled water with decresing pH around 4.2 using 4N HCl treatment. The treated samples were incubated at room temperature (24-26°C) for 7 days to hydrolyze fish protein by activing endogenous enzymes. After incubation period, the hydrolyzed samples, then treated at 85°C for 20 minutes to inactive enzymes, followed by centrifugation at 5000 rpm for 10 minutes to separate samples into three fractions, namely solid phase, liquid phase and oil phase. The liquid phase compound was then spray-dried at inlet temperature of 160°C and an outlet temperature of 90°C. The obtained peptone was stored at low temperature until used.

Characterization of mackerel peptone

Determination of chemical composition: Total nitrogen, fat, moisture, and ash contents were determined by the method of AOAC (2005). Total nitrogen was determined by the Kjeldahl method. Fat content was determined using Soxhlet method. For moisture content, gravimetric method was used in this study. In addition, ash content of extracted peptone was analyzed by the gravimetric method.

Determination of solubility: Solubility was analyzed using the gravimetric method (Ningsih et al., 2018). About 1 g sample dissolved in 150 mL of distilled water. The solution then filtered using Whatman paper no. 2. Solubility can be determined using the formula:

$$\text{Solubility } (\%) = \frac{100-(a-b)}{(100-MC)\times c} \times 100 \quad (1)$$

Where, a = Whatman paper plus residue

b = initial weight of Whatman paper

c = sample weight

MC = moisture content

Table 1. Formulation of culture media.

Ingredient	Positive control	Negative control	Mackerel
Commercial peptone	10.00	-	-
Mackerel peptone	-	-	10.00
Yeast extract	5.00	5.00	5.00
Sodium chloride	10.00	10.00	10.00

Determination of color parameter: The determination of color parameter of peptone extracted from mackerel head by-product with acid-adding treatment was measured using a Konica minolta chroma meter CR-400 (Japan). The color analysis were catagorized into L*, a*, and b* values. The L* value means lightness, the a* value means greeness to redness, and the b* value means blueness and yellowness. The instrument was calibrated with the standard before used in determining color. After calibrating, the peptone sample was put into petri dish, then, the sensor section pointed at the peptone sample and noted the results of color test consist of L*, a*, b*, whiteness and color intensity.

Bacterial Growth Test: Preparation of culture media: Growth media formulations consist of peptone extracted from mackerel head by-product, commercial peptone, sodium chloride and yeast extract, which were equivalent to the composition of Luria Bertani (LB) broth. Media sterilized using autoclaves at 121°C for 15 minutes at a pressure of 1 atm (Andualem and Gessesse, 2013). To obtain the growth performance of each peptone tested in this study, the formulation of culture media was used varies as follows:

Bacterial growth measurement: The Optical Density (OD) was analyzed by testing the growth of *Escherichia coli, Staphylococcus aureus, Salmonella thyphi and Aeromonas hydrophila* on each media. Incubation was done using a shaker with a speed of 100 rpm at 3^{5}oC. Bacterial growth measured at 0-36 hours every 6 hours and measured using a spectrophotometer with a wavelength of 600 nm (Shirahigue et al., 2018).

Biomass production: Biomass production was determined at 24-hour growth. 25 ml sample containing bacteria and media was transferd into a sterile falcon tube then centrifuged at 5000 rpm for 20 minutes. The precipitate was separated, then, added 5 mL of 0.85% NaCl solution (w/v). Afterward, the precipitated sample was centrifuged in the same condition. Finally, the precipitate was dried for 24 hours at 105°C (Poernomo and Buckle, 2002). Statistical Analysis: Data were examined using analysis of variance (ANOVA), and in the case of significant

difference was signed as $p<0.05$, followed by a Tukey's post-hoc test using SPSS 25.0 software.

RESULTS AND DISCUSSION

Chemical Composition: The chemical analysis of peptone extracted from mackerel head by-product was observed, and the results are presented in Table 2. The chemical composition of mackerel peptone consisted of total nitrogen, fat, moisture, and ash accounted for 11.53%, 1.33%, 5.07%, and 3.78%, respectively. The peptone extracted from mackerel head by-product was higher in term of nitogen content than that of fish by-product peptone reported by Najim et al. (2015) and it was similar to yellowstripe sead fish peptone (Saputra and Nurhayati, 2013). However, when compared to commercial peptone, the nitrogen content of mackerel peptone was lower than that of commercial peptone. The peptone isolated from mackerel head by-product was higher in fat and moisture content than those of fish by-product peptone and commercial peptone reported by Najim et al. (2015), while the ash content of mackerel peptone was lower when compared to those of peptone mentioned above. It might be indicated that component of peptone (expecially nitrogen content) extracted from mackerel head by-product was still crude compared to the commercial peptone. In addition, the total nitrogen is influenced by the protein content and the hydrolysis process in the raw material (Barokah et al., 2017). Total nitrogen obtained from the peptide bond breaking process becomes simpler during the hydrolysis process and the hydrolysis process is also influenced by various factors such as pH, temperature, and time (Ningsih et al. 2018). However, the mackerel peptone could be used as a essential substrate of bacterial growth media.

Table 2. Chemical characteristics of mackerel head, fish head by-product and commercial peptones.

Parameter (%)	Mackerel head peptone	Fish by-product peptone*	Commercial peptone**
Total Nitrogen	11.53	10.8	15.10
Fat	1.33	0.8	0.4
Moisture	5.07	3.1	1.5
Ash	3.78	8.0	5.0

* reported by Najim et al. (2015)
** NeoGen Lab peptone

Color Parameter: The color parameter of the peptone isolated from mackerel head by-product was measured and the results are tabulated in Table 2. and depicted in Fig. 1. The L*, a* and b* value of the mackerel peptone were 85.11%, 0.04%, and 19.04%, respectively. When compared to commercial peptone, the L*, a*, and b* value of mackerel peptone was lower than that of commercial peptone, indicating the color of mackerel peptone (87%) showed slightly lower intensity than that of commercial peptone (90.71%). However, the whiteness of peptone extracted from mackerel head by-product was higher compared to commercial peptone, which was 48.94% in the mackerel peptone, and 40.51% in the commercial peptone. Research has reported that peptone extracted from fish by-products (multi-species of marine fish) has color parameter consists of 52.64 in L*, 2.50% in a*, 7.99% in b* and 51.44% in whiteness (Nurhayati et al. 2015), and Barokah et al. (2017) investigated that microencapsulated peptone from marine fish by-product performed the L*, a*, b*, and whiteness value accounted for 60.01%, 1.70%, 10.33% and 57.44%, respectively.

Solubility: Solubility of peptone is an indicator to determine the quality of peptone, as stated by Khalil (2012), peptone is protein hydrolysates that are soluble in water and not coagulable by high temperature. The peptone extracted from mackerel head by-product had solubility around 97.07%, which was lower solubility when compared to that of commercial peptone with solubility around

Table 3. Color parameter of mackerel and commercial peptone

Parameter	Mackerel peptone	Commercial peptone*
Lightness (L*)	85.11±0.06	86.45±0.06
Green-red (a*)	0.04±0.05	0.59±0.06
Blue-yellow (b*)	19.04±0.06	27.48±0.04
Whiteness	48.94±0.07	40.51±0.05
Color intensity	87±0.05	90.71±0.06

*NeoGen Lab peptone

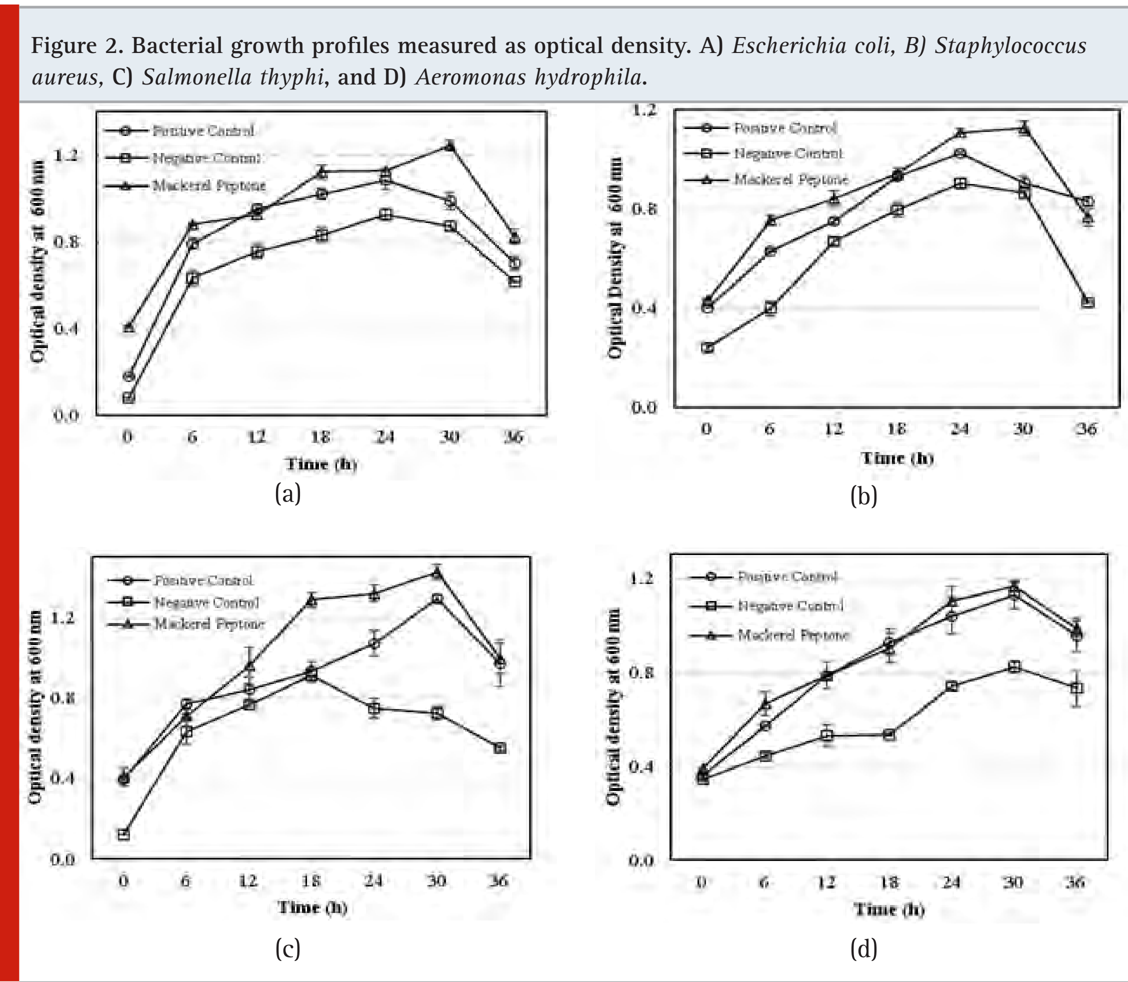
100%. The solubility of mackerel peptone in accordance with the solubility of yellowstripe sead fish peptone reported by Saputra and Nurhayati (2013). The high solubility value of peptone product is due to the presence of a hydroxy group in the mackerel peptone that interact with water molecules (Ningsih et al. 2018). High solubility value in protein hydrolyzate is caused by the reaction of protein breakdown into smaller peptides (Barokah et al. 2017).

Figure 1. Color. (a) Commercial peptone (NeoGen Lab) and (b) Mackerel peptone

(a) (b)

Bacterial Growth Profile: Four different bacterial species (*Escherichia coli, Staphylococcus aureus, Salmonella thyphi* and *Aeromonas hydrophila*) were selected to determine the growth rate in the Luria-Bertani (LB) broth with some modifications. The growth profiles of both mackerel and commercial peptones can be shown in Fig. 2. The bacteria grew properly in the LB broth supplemented with the mackerel peptone, as well as commercial peptone. The results depicted in Figs. 2A-2C, almost all the bacterial growth rates supplemented the mackerel peptone showed better performance, compared to commercial peptone (as a positive control), as well as untreated peptone (as a negative control) during the research period. However, only for *Aeromonas hydrophila*, the growth rate of peptone isolated from mackerel

Figure 2. Bacterial growth profiles measured as optical density. A) *Escherichia coli, B) Staphylococcus aureus,* C) *Salmonella thyphi*, and D) *Aeromonas hydrophila*.

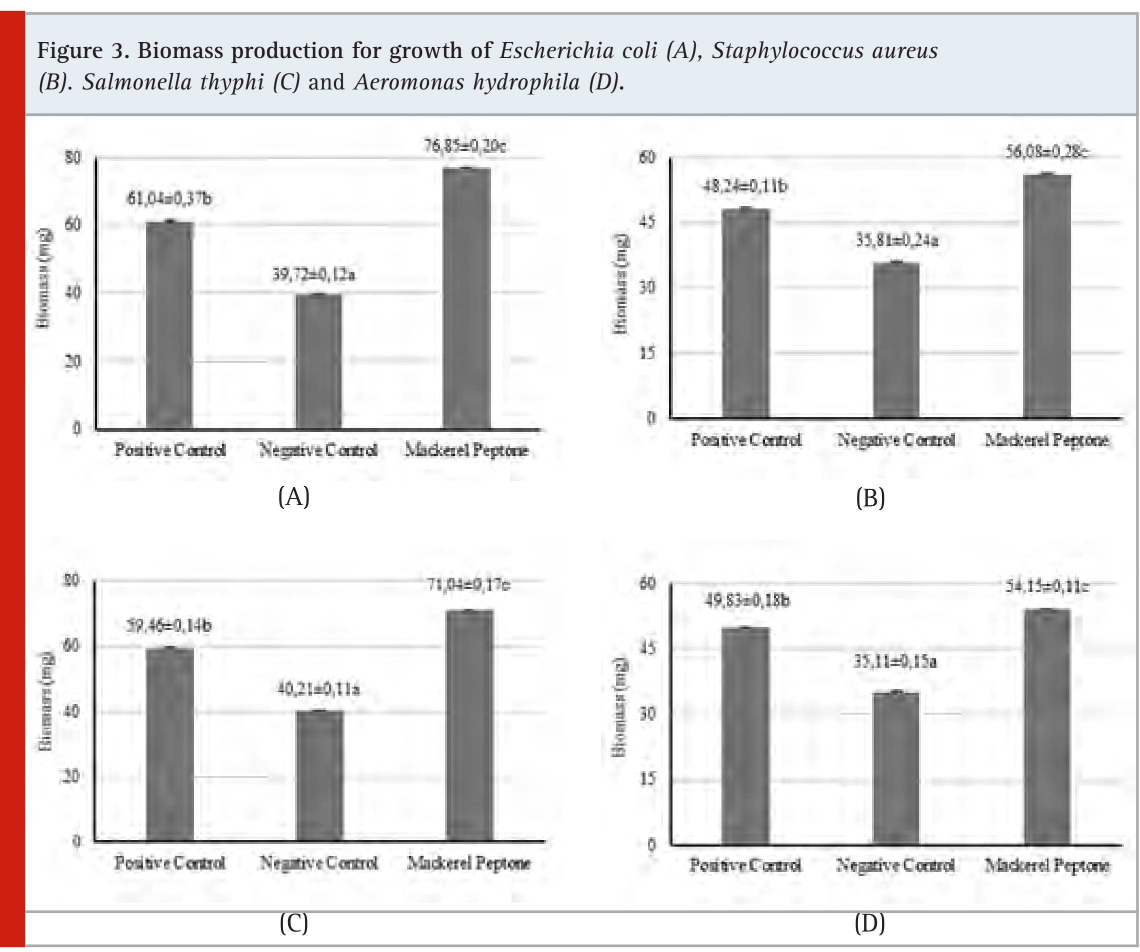


head by-product was in the line with commercial peptone, but both of them grew faster than that of the untreated peptone.

This might be suggested that the peptone isolated from mackerel head by-product is more effective for supporting growth of bacteria in the LB-broth media than that of commercial product. Previous studies have investigated that peptones extracted from different fish species, such as cod, salmon, tuna, and unspecified fish showed more higher microbial growth rate than that of a casein peptone (Dufossé et al., 2001). Peptones from cowtail ray (*Trygon sephen*) have superior performance in supporting different species of microorganims (*Aspergillus flavus, Bacillus subtilis, Saccharomyces cerevisiae, Escherichia coli*, and *Staphylococcus aureus*) were compered to commercial peptones (Poernomo and Buckle, 2002). In additon, peptones isolated from different fish species performed better bacterial growth profile than that of commercial peptones (Vieira et al., 2005; Safari et al., 2011). Nevertheless, Najim et al. (2015) reported the fish by-product peptones have lower performance when compared to commercial peptone for *Pseudomonas aeruginosa, Lactobacillus acidophilus,* and *Saccharomyces cerevisiae.*

Biomass Production: The bacterial biomass production of peptone produced from mackerel head by-product was observed and the results are illustrated in Fig. 3. The biomass production of all bacterial cultures consisted of *Escherichia coli, Staphylococcus aureus, Salmonella thyphi* and *Aeromonas hydrophila* ranged from 30.21-60.32, 30.21-60.32, 30.21-60.32 and 30.21-60.32 (in mg per 100 mL), respectively. For the culture of *Escherichia coli*, the biomass production in the medium supplemented by the mackerel peptone showed significantly increase ($p<0.05$) in comparison with either commercial peptone (+ control) and no added peptone (- control). This result was also similar to the cultures of

Figure 3. Biomass production for growth of *Escherichia coli (A), Staphylococcus aureus (B). Salmonella thyphi (C)* and *Aeromonas hydrophila (D).*



Staphylococcus aureus and Salmonella thyphi, which the mackerel peptone presented higher bacterial biomass yields than those in commercial and no supplemented peptones. However, the culture of *Aeromonas hydrophila* yielded a significantly increase ($p<0.05$) of biomass production in the commercial peptone than those in mackerel peptone and negative control.

This might be indicated that the peptone produced from mackerel head by-product was effective for supporting bacterial growth in terms of biomass by providing the appropriate sources of nitrogen. Poernomo and Buckle (2002) stated that the high biomass production provides adequate nutrients in supporting the growth rate of microorganisms. In addition, the higher biomass production obtained, the more effective growth rate of media supplemented with peptones as a microbial substrate. These findings are in the line with some studies revealed that peptones produced from different fish by-products, such as *Panulirus argus*, *Panulirus laevicauda*, and *Macrobrachium amazonicum* showed a significant biomass production of *Escherichia coli* compared to commercial peptone (OXOID) (Vieira et al., 2005). Moreover, Poernomo and Buckle (2002) and Shirahigue et al. (2018) reported higher result of biomass yield by five microorganisms in Trygon sephen peptones and by two bacteria (*Escherichia coli* and *Staphylococcus aureus*) in both *Oreochromis niloticus* and *Rachycentron canadum*, respectively.

CONCLUSION

In summary, peptone extracted from mackerel head by-product was characterized by chemical composition, solubility, color parameter, bacterial growth, and biomass production. Though the nitrogen compound, solubility and color intesity of mackerel peptone were lower than that of commercial peptone, almost the bacterial growth rates and biomass productions for *Escherichia coli, Staphylococcus aureus, Salmonella thyphi,* and *Aeromonas hydrophila* showed significantly faster and higher in mackerel peptone compared to the commercial peptone. Thus, peptone produced from mackerel head by-product can be an alternative source of as halal substrates for bacterial growth.

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Endomycorrhizae Enhances Reciprocal Resource Exchange Via Membrane Protein Induction

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ABSTRACT

Endomycorrhizae have the ability to exploit organic sources while providing their host plant with nitrogen and phosphorus, but the mutualistic molecular mechanism by which this occurs is unclear. In a previous study, five uncharacterized root proteins were found to be significantly upregulated in endomycorrhiza-colonized sorghum, which showed an increase in biomass and element uptake. However, the functions of these proteins are unknown. Therefore, in the current study, the functions of these five proteins were explored using computational analysis. Since these root proteins were not identified in plant protein sequences, they were submitted to Blast to search for homologous fungal protein data. The possible functions of the resulting 406 homologous proteins were investigated by performing several analyses, namely, phylogenetic analysis, sequence similarity network (SSN) analysis, genome neighborhood network (GNN) analysis, and functional network identification. The results of analyzing the uncharacterized sorghum root proteins revealed three integral membrane proteins among them: an APC amino-acid permease, a transmembrane transporter activity protein, and an acid protease. These results emphasize the role of endomycorrhiza–plant interactions in increased root permeability, resulting in the enhanced exchange of reciprocal resources in a symbiotic process.

KEY WORDS: *ARBUSCULAR MYCORRHIZA, MUTUALISM, ENDOMYCORRHIZA, SORGHUM, ROOTS, MEMBRANE PROTEINS*

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INTRODUCTION

The health of the biosphere, which largely depends on plant and environmental factors, shapes the interactions among dynamic communities involving macro- and microorganisms, such as bacteria and mycorrhizas. An arbuscular mycorrhiza (AM), also known as endomycorrhiza, colonizes and branches within the root cells of plants and stores oil-rich products in its vesicles. After successful colonization, the AM grows a branch-like structure (known as hyphae) that extends outside the roots for several centimeters in the soil. Hyphae facilitate nutrient uptake, particularly phosphate ions and nitrogen, in a tradeoff for carbon extraction from plant roots (Coleman et al., 2017). The beneficial effects that the AM confers to the plant occur across diverged membrane interfaces in a symbiotic relationship between the fungus and the host plant (Casieri et al., 2013). The development of AM colonization requires the plant cell's secretory mechanism to improve the rapid branching of fungal hyphae and generate a periarbuscular membrane (Ivanov et al., 2019).

Extensive studies have focused on the influence of AMs on plant growth and biomass, nutrient availability, and metabolites (Dhawi et al., 2015; Dhawi et al., 2016; Dhawi and Hess, 2017a, b; Dhawi et al., 2018).The use of an AM alone or in combination with plant-growth-promoting bacteria was found to enhance the activity of the radical scavenging system in sorghum roots (Dhawi et al., 2017) and contribute to the upregulation of the metabolites associated with amino acid synthesis in foxtail millet (Dhawi et al., 2018). Many studies have suggested that the symbiotic process involves rhizobiome assembly governed by novel protein families (Sasse et al., 2018). One study reported the upregulation of 82 proteins in the plasma membrane of plants in response to mycorrhizal colonization (Aloui et al., 2018); thus, the plant's carbohydrate and nitrogen-derived transporters were enhanced during AM colonization (Hacquard et al., 2013). Although previous studies have provided insights into the system of rhizosphere microbiomes, little is known about plants' molecular changes during colonization. Here, we reveal the function of five uncharacterized sorghum proteins in roots treated with endomycorrhiza. In a previous study, endomycorrhiza colonization resulted in an increase in sorghum root biomass and element uptake, together with a significant upregulation of five proteins with unknown functions (Dhawi et al., 2017).

Table 1. Fold changes in uncharacterized sorghum proteins influenced by endomycorrhiza

Protein ID	Fold change compared with control
C5XHS5	33
C5X3P2	20
C5Y9B5	16
C5XV25	13
C5XHR8	11

In this study, the functions of these unknown proteins were explored using computational analysis. Several steps were taken to identify the functions of these proteins. The sequences of the uncharacterized sorghum root proteins were submitted to Blast to search for homologous protein sequences in fungal protein data since they were not identified in plant protein sequences. This resulted in the identification of 406 homologous proteins, which were then subjected to several analyses to determine their possible roles. These analyses included sequence similarity network (SSN) and genome neighborhood network (GNN) construction, as well as functional network identification. The results of analyzing the uncharacterized proteins revealed three integral membrane proteins: APC amino-acid permease, transmembrane transporter activity protein, and acid protease. These findings add to our understanding of the molecular mechanisms by which mycorrhizal synergetic activity enhances plant productivity.

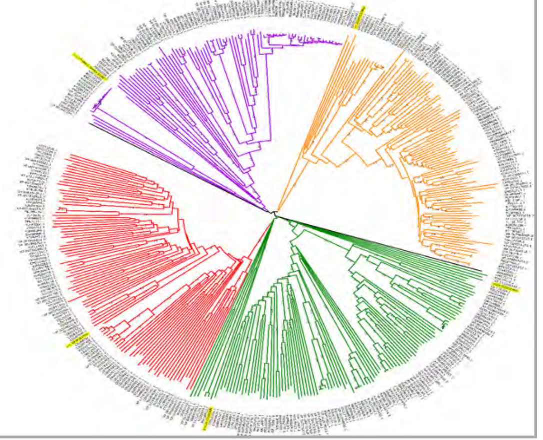
MATERIAL AND METHODS

Plant growth conditions and treatments: Two-day-old germinated seedlings of Sorghum bicolor (BTx623, USDA) were planted in 600 g of pasteurized soil mix consisting of coarse sand and loam soil (1:1). The soil mix was inoculated with 9 g (~1320 propagules) of an endomycorrhizal

mix (MycoApply® Endo, Valentine Country Inc, USA), and the control group was inoculated with 9 g of a pasteurized endomycorrhizal mix at 70 °C (Dhawi et al., 2015). The endomycorrhizal mix consisted of ~1320 spores of Glomus intraradices, G. mosseae, G. aggregatum, and G. etunicatum. Sorghum roots were harvested 90 days after treatment and subjected to protein extraction and identification. Protein was extracted from frozen roots. Three samples from each group were processed following the procedure described in Dhawi et al. (2017) and modified in Fukao et al. (2011).

Protein processing of uncharacterized sorghum roots: The sequences of the five uncharacterized sorghum root proteins with increased fold changes (11–33-fold) (Table 1) were used to search for homologous fungal proteins using PSI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (Altschul et al., 1997), which resulted in 401 protein sequences. These sequences were filtered using CD-HIT Suite: Biological Sequence Clustering and Comparison (http://weizhongli-lab.org/cdhit_suite/cgi-bin/index.cgi), with a 90% identity cut-off. The 90% cut-off step resulted in 406 protein sequences for performing a multiple sequence alignment (MSA). The MSA was created using the MEGA7 (Molecular Evolutionary Genetics Analysis) software version 6 (Tamura et al., 2013). The phylogenetic tree was established in MEGA6 and aligned using the Multiple Sequence Alignments (MUSCLE) program (Edgar, 2004) for 406 proteins, five of which were the uncharacterized upregulated sorghum root proteins. Protein sequences were aligned using the neighbor-joining method (Saitou and Nei, 1987) and UPGMA (unweighted pair group method), with 500 bootstrap replications for the phylogenetic assessment. The interactive tree of life (iTol) online web tool (http://itol.embl.deg) was then used to display and modify the tree colors (Letunic and Bork, 2016).

Figure 1. The phylogenetic tree of five uncharacterized sorghum proteins (highlighted in yellow, C5XHS5, C5X3P2, C5Y9B5, C5XV25 and C5XHR8) and similar fungal proteins. The unrooted neighbor-joining phylogenetic tree was constructed using the sequence alignment of 406 multiple proteins using Multiple Sequence Alignments (MUSCLE) with 500 bootstrap replicates



Protein functional network and family assignment: A combination of different approaches was used to assign homologous proteins to protein families (Pfam) with putative functions. The 406 protein sequences consisted of uncharacterized sorghum root proteins and similar fungal protein sequences used to find their functions on the basis of their genomic, protein, and gene interactions using the sequence similarity network (SSN) and genome neighborhood network (GNN) approaches. The GNN approach was performed using the Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST) (https://efi.igb.illinois.edu/efi-est/; Gerlt et al., 2015).

For EFI-EST, the 406 proteins were first converted to UniProt IDs. Only 60 proteins were identified in the UniProt database, and 346 were unidentified fungal proteins. The EFI-EST website was used to generate an SSN, and the resulting files were visualized using the Cytoscape software (Shannon et al., 2003). Sorghum protein sequences were screened for motifs using the MEME (Multiple EM for Motif Elicitation) tool (http://meme-suite.org/tools/meme) (Tanaka et al., 2011), with the detection level set to a maximum of eight motifs and an E-value equal to or less than 0.05.

RESULTS AND DISCUSSION

Phylogenetic tree analysis: The phylogenetic tree of 406 protein sequences contained five uncharacterized sorghum proteins and 401

similar fungal proteins aligned using Multiple Sequence Alignments (MUSCLE) with 500 bootstrap. The resulting unrooted phylogenetic tree consisted of 406 nodes, in which sorghum uncharacterized protein sequences clustered in five of them (Fig.1). Phylogenetic tree analysis identified uncharacterized sorghum protein C5XHS5 clustered in one clade with fungal protein of ORZ25315.1, which belonged to Amino acid/polyamine transporter I. However, sorghum protein C5Y9B5 was clustered with four fungal proteins, namely 'XP 013245342.1, EDK37078.2, TDL21399.1 and OAX33366.1. C5XHR8 clustered with AWI66956.1 and CEI935941.

On the other hand, two sorghum proteins were sorted with no cluster, with fungal proteins C5XV25 and C5X3P2 (Fig.1). The sorghum uncharacterized root protein C5XHS5 which was upregulated to 33 fold and showed to be close to fungal protein ORZ25315.1 based on phylogenetic analysis. This protein belongs to the Absidia repens protein that functions as transmembrane transporter activity (https://www.uniprot.org/uniprot/A0A1X2J0L6). The second sorghum protein was C5Y9B5, which showed to be close to all these fungal proteins : XP 013245342.1, which belongs to Tilletiaria anomala that functions as Acid protease (https://www.uniprot.org/uniprot/A0A066WIX7); EDK37078.2, which belongs to uncharacterized protein of yeast Meyerozyma guilliermondii (https://www.uniprot.org/taxonomy/294746); TDL21399.1, which belongs to uncharacterized protein of fungus Caballeronia sordidicola (https://www.uniprot.org/uniprot/A0A4R1P5H9) and close to OAX33366.1 protein of fungus of Rhizopogon vinicolor, which functions as Acid protease (https://www.uniprot.org/taxonomy/1314800). Furthermore, C5XHR8 clustered with fungal protein of AWI66956.1 belongs to fungal proteins of Neocallimastix cameroonii and known as the Glycosyl hydrolase family 17 (https://www.uniprot.org/uniprot/A0A2S1TZ75) and with CEI93594.1 belongs to uncharacterized protein of fungus Caballeronia sordidicola (https://www.uniprot.org/uniprot/A0A0A1NM33).

Sequence similarity network (SSN): The SSN is used to analyze 60 sequences containing five sorghum uncharacterized protein sequences and 55 fungal protein sequences. These sequences were obtained by PSI-BLAST at E.value 10-15; the sequences were clustered based on their similarities in four clusters. The SSN clusters contained one uncharacterized sorghum protein, whereas, the other four proteins were separated with no clusters due to a different E.value score.

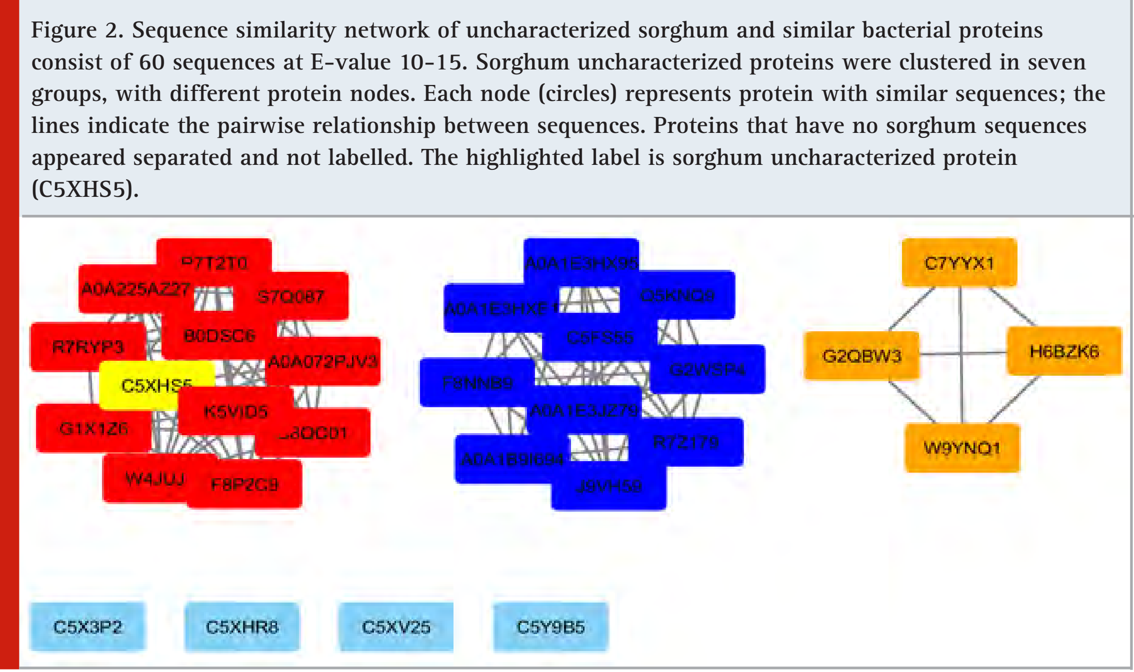
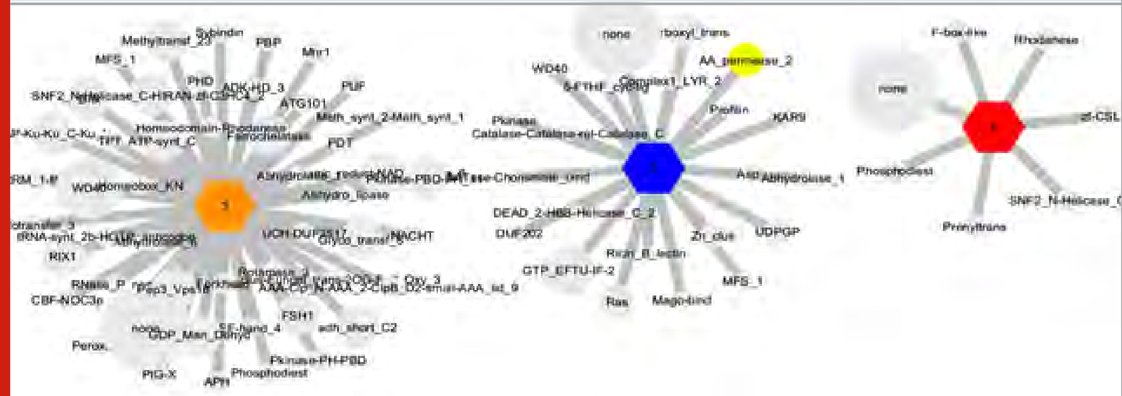


Figure 2. Sequence similarity network of uncharacterized sorghum and similar bacterial proteins consist of 60 sequences at E-value 10-15. Sorghum uncharacterized proteins were clustered in seven groups, with different protein nodes. Each node (circles) represents protein with similar sequences; the lines indicate the pairwise relationship between sequences. Proteins that have no sorghum sequences appeared separated and not labelled. The highlighted label is sorghum uncharacterized protein (C5XHS5).

Figure 3. Genome Neighborhood Networks (GNNs) in Hub-Nodes format for 76 protein sequences. The hexagon shapes (hubs) represent SSN clusters and the other shapes (nodes) represent the Pfam genome neighbors. Three node clusters are indicated with 76 genes. The names of the nodes are the short name for protein family (Pfam). The highlighted pfm is associated with sorghum uncharacterized protein (C5XHS5)



These resulting SSNs were used to establish Genome Neighborhood Network (Fig. 2). Genome Neighborhood Networks GNN, and functional partners: The SNN results are used to build GNNs. The output of GNN in Hub-Nodes format showed the number of Pfam gene neighbors that have been found in each cluster of sequences. The SNNs of uncharacterized protein sequences of sorghum were clustered in seven nodes. The nodes were protein families, arranged based on their genomic context and shared 86 protein families (Fig.3). The resulting SSNs were used to establish Genome Neighborhood Network identified nodes of protein families, arranged based on their genomic context. The protein family identified showed several forms in KEGG, namely: (https://www.genome.jp/dbget-bin/www_bget?lbc:LACBIDRAFT_191715) ,(https://www.genome.jp/dbget-bin/www_bget?gtr:GLOTRDRAFT_140033) and (https://www.genome.jp/dbget-bin/www_bget?hir:HETIRDRAFT_66031). On the other hand, the analysis of uncharacterized sorghum root proteins identified one protein family, which was Amino acid permease (Pfm: PF13520) (Fig. 3).

The nature of the mutualistic interaction between plants and endomycorrhiza has been extensively studied. However, endomycorrhiza symbiotic proteins and signaling molecules have remained unexplored. In the current study, the uncharacterized sorghum root protein C5XHS5, which was upregulated by 33-fold, was shown to be close to fungal protein ORZ25315.1—an Absidia repens protein that has transmembrane transporter activity. C5Y9B5 was shown to be close to two fungal proteins: one belongs to *Tilletiaria anomala*, and the other protein belongs to the fungus Rhizopogon vinicolor. Both of these proteins function as an acid protease. C5XHR8 clustered with the fungal protein of Neocallimastix cameroonii known as glycosyl hydrolase family 17. One uncharacterized sorghum protein was grouped in SSN clusters, whereas the other four proteins were separated without clusters because of their different E-value scores.

The only uncharacterized sorghum protein that was identified was C5XHS5. This protein was especially upregulated (33-fold) in sorghum roots after endomycorrhizal colonization, and it clustered with 11 fungal proteins. Next, the resulting SSNs were used to establish a genome neighborhood network, which identified one protein family, namely, APC amino-acid permease. Arbuscular mycorrhizal (AM) symbiosis, which is assumed to enhance water and nutrient absorption, is associated with massive arrays of membrane tubules that form between the plant protoplast and the cell wall (Ivanov et al., 2019). The plasma membrane-associated proteins that respond to mycorrhization support the host

plant's control of sugar uptake and mediate the replacement of phospholipids by phosphorus-free lipids in the plasmalemma of mycorrhizal roots (Aloui et al., 2018). This explains the increase in carbohydrate and nitrogen-derived transporters during the endomycorrhiza's symbiotic stages since these transporters enhance the transfer of reciprocal resources between the host and the symbiont (Hacquard et al., 2013). The uncharacterized sorghum root proteins that were highly upregulated are part of the protein families that function as a transmembrane transporter, acid protease, and APC amino-acid permease. This result is similar to that of a previous study on amino-acid permease that described this protein as a molecular tool of endomycorrhiza (Glomus mosseae).

This study identified an increase in amino-acid permease following exposure to organic nitrogen from the soil, thereby enhancing amino acid acquisition (Cappellazzo et al., 2008). The amino acid transporter (AAT) gene (LjLHT1.2) that encodes for the lysine–histidine–transporter (LHT)-type amino acid transporter induced in mycorrhizas is involved in the complex mechanisms of amino acid recycling in colonized roots (Guether et al., 2011).

CONCLUSION

The analyses of five significantly upregulated proteins in endomycorrhiza-colonized sorghum roots add to our understanding of the complex molecular mechanisms of mycorrhizal synergetic activity. The enhancement of plant productivity is the result of the mycorrhizal-induced upregulation of plant membrane proteins and the expression of the associated genes, thereby increasing root permeability and reciprocal resource exchange between the host and the symbiont.

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Does Prediabetic State Affects Dental Implant Health? A Systematic Review And Meta-Analysis

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ABSTRACT

Antimicrobial The aim of the present communication is to evaluate whether prediabetic state affects peri-implant health status? Several databases were searched from August 1975 up to August 2019 for studies that evaluated the clinical and radiographic peri-implant parameters including plaque index (PI), bleeding on probing (BOP), probing depth (PD), and crestal bone level (CBL) in patients with prediabetes and non-diabetes. The standard mean differences (SMD) of outcomes and 95% confidence intervals (CI) for each variable were calculated using random effect model. Quality assessment and risk of bias were estimated using ROBINS-I and GRADE. Six studies were included. An overall quality assessment showed low to moderate risk of bias. The overall weighted mean difference for PI (SMD=3.42, 95% CI= 0.67 to 6.17, P=0.015), BOP (SMD=6.69, 95% CI= 4.94 to 8.45, P<0.001), PD (SMD=7.77, 95% CI= 4.87 to 10.67, P<0.001) and CBL (SMD=6.87, 95% CI= 0.98 to 12.77, P=0.023) showed statistically significant differences between prediabetes and non-diabetic groups, respectively. The direction of recommendation emerging from this meta-analysis is strong in favour of prediabetes in the deterioration of peri-implant health compared to non-diabetic patients.

KEY WORDS: PREDIABETIC STATE; PERI-IMPLANTITIS; DENTAL IMPLANTS; SYSTEMATIC REVIEW.

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INTRODUCTION

According to American Diabetes Association, type-2 diabetes mellitus (T2DM) is defined as impaired blood glucose levels that is associated with either due to deficiency of insulin or simply its altered function, (Association, 2018). Significant amount of research indicates that dental implant therapy could be a viable treatment option if patients maintain their serum glycated haemoglobin (HbA1c) levels, (Ormianer, Block, Matalon, & Kohen, 2018; Vissink, Spijkervet, & Raghoebar, 2018). However, modest amount of data exists that describes the associations between peri-implant health and moderate glycemic conditions including well-controlled diabetes mellitus and prediabetes.

Prediabetic state is described by the elevated blood glucose levels that are nonetheless below the threshold for apparent diabetes, (Association, 2010; Organization, 1999). Prediabetes may be recognized by either of the two conditions such as impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) which are extremely prevalent in the developed countries. (Atlas) Studies have suggested that patients with prediabetes show poor clinical periodontal parameters compared to the non-diabetic counterparts, (Javed et al., 2012; Javed et al., 2014 and Alasqah et al., 2018). This accounts with the persistent hyperglycemia in prediabetic state that establishes an imbalance between destructive periodontal pathogens and the host immune response,(Demmer et al., 2015).

Which further leads to the formation and accumulation of advanced glycation end-products (AGEs), proinflammatory cytokines, and dysfunction of polymorphonuclear neutrophils, thereby leading to the breakdown of supporting soft tissues and alveolar bone, (Takeda et al., 2006, Andriankaja & Joshipura, 2014). Similar mechanisms may also be involved in peri-implant health, however there is no agreement on this subject. Although a significant amount of implant therapy is performed in routine oral health practice, however, it is imperative to ascertain patients who are supposedly at greater risk of oral complications, including dental implant failure due to periimplant diseases, (Mombelli & Cionca, 2006 and Bornstein, Cionca, & Mombelli 2009). Reflecting the rising concerns regarding the high prevalence of prediabetes globally and the increasing amount of patients seeking dental implant treatment, the purpose of this meta-analysis was to gather and summarise all empirical evidence on the potential association between prediabetes and dental implant health and its complications.

This systematic review presents the following null hypotheses. Firstly, no significant differences are observed in implant survival rate between patients with prediabetes and those who are non-diabetic. Secondly, no significant differences are observed between these groups with regards to clinical and radiographic peri-implant parameters including peri-implant probing depth (PD), bleeding on probing (BoP), or crestal bone level (CBL) around dental implants. The aim of the present study was designed to answer the PECOS (Patients; Exposure; Comparators; Outcomes; Study design) question. The focused PECOS clinical question of the present study was: Does prediabetic state (exposure) affects peri-implant clinical parameters (outcomes) considering the outcomes were assessed in retrospective and/or prospective studies (study design)?

MATERIAL AND METHODS

Protocol and eligibility criteria: This systematic review and meta-analysis followed the guidelines described by PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis),(Moher, Liberati, Tetzlaff, Altman, & Group, 2009) and followed the outlines of PECOS,(Schardt, Adams, Owens, Keitz, & Fontelo, 2007). Cross-sectional data gathered in either retrospective, case-control or prospective study design were considered. Clinical or radiographic peri-implant parameters including PI, BOP, PD and CBL reported in human subjects with and without prediabetes (according to American Diabetes Association)(Chamberlain, Rhinehart, Shaefer, & Neuman, 2016) were included. In addition, the present study only considered studies in English language. Studies with missing data on both PD and CBL were excluded. Also, in-vitro, comprehensive reviews, experimental studies, abstracts, case-series were excluded.

Systematic literature search: Main databases (EMBASE, MEDLINE, COHGTR and CENTRAL) were searched between August, 1975 and August, 2019 using the following terms: ((Prediabetic state) OR (prediabetes) OR (impaired fasting glucose) AND ((peri-implant) OR (peri-implantitis) OR (peri-implant diseases) AND (plaque) OR

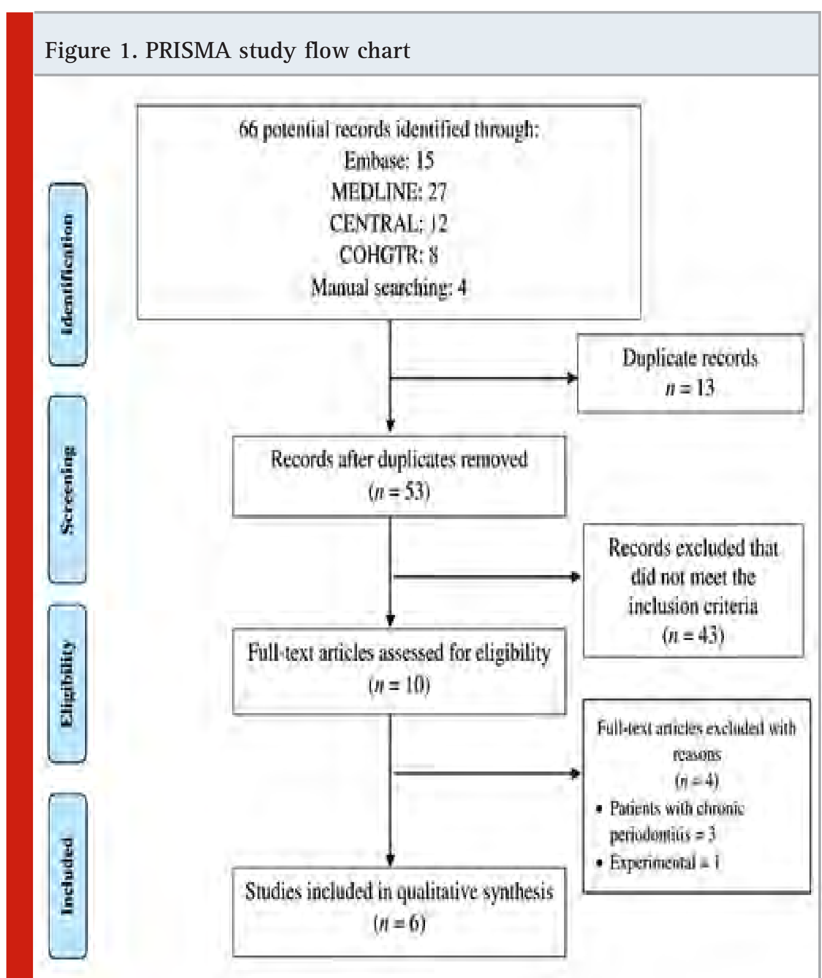
(plaque scores) OR (plaque index) OR (bleeding on probing) OR (radiographs) OR (crestal bone loss) OR (marginal bone loss) OR (peri-implant bone loss). After reading the main titles and abstracts, their eligibility for inclusion in the study were judged. Once the complete list of included articles was gained, their complete texts were downloaded for subsequent data abstraction and assessment. Studies overlooked from electronic search database were subsequently manually searched in the following Web of Science journals including Clinical Implant Dentistry and Related Research, Clinical Oral Implants Research, and Acta Odontologica Scandinavica.

Published studies that satisfied the inclusion criteria were handled for data abstraction. The intent of the project was to comply with the standards set in the PRISMA guidelines. Following this, the details from the included articles were tabulated according to the study designs, level of evidence as described by the Oxford Centre for Evidence-Based Medicine (CEBM) (Howick et al., 2009), patient data, glycemic status, duration of prediabetes, covariates, parameters of peri-implant health, and final conclusions. Data was gathered and summarized according to the PECOS question.

Risk of bias in individual studies: This study evaluated the quality of included studies using the Risk Of Bias In Non-randomised Studies (ROBINS-I) assessment tool.(Sterne et al., 2016) Five important domains that estimates bias includes potential confounders, selection, classification, attrition and reporting bias are assessed in this tool. Each of the sections could be given one of the following ratings: 'low risk', 'moderate risk', 'serious risk', 'critical risk', or 'no information'.

Risk of bias across studies using GRADE approach:

Figure 1. PRISMA study flow chart



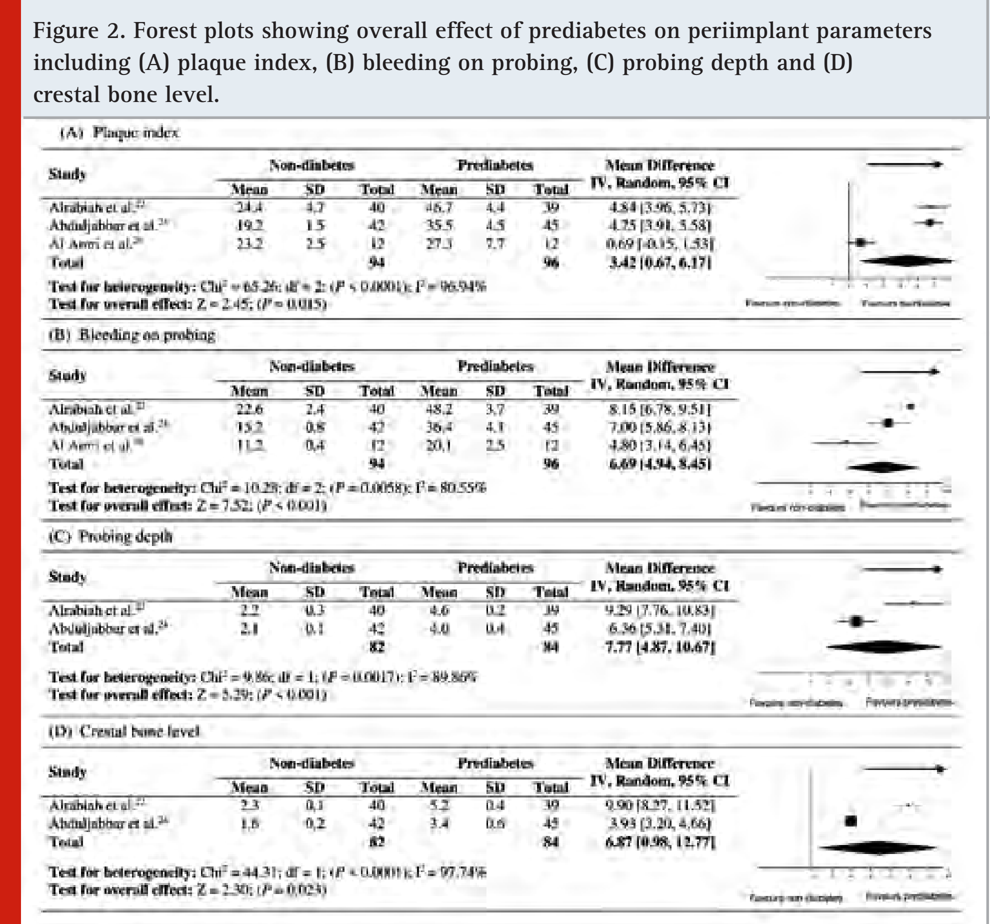
Evaluation for the overall quality of evidence was conducted according to the Grades of Recommendation, Assessment, Development and Evaluation (GRADE).(Guyatt, Oxman, Schünemann, Tugwell, & Knottnerus, 2011). These are based on the following scores: 'high quality': we are very assertive that the real effect lies close to that of the estimate of the effect, 'moderate quality': we are moderately assertive in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different, 'low quality': our assurance in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect, and 'very low quality': we have very little assurance in the effect estimate: the true effect is likely to be markedly different from the estimate of effect.

Meta-analysis: Literature that reported data on clinical and radiographic peri-implant parameters were processed for data synthesis. I2 and $\chi 2$ statistics were applied for estimation of heterogeneity. Depending on the degree of heterogeneity, either random model or fixed models were used in case of heterogeneity being significant (I2 >50%) or being low (I2 ≤50%), respectively,(Borenstein, Hedges, Higgins, & Rothstein, 2010). P-value was set at 0.05 that represented statistical significance. Forest plots were generated explaining standard mean difference (SMD) of outcomes with 95% confidence intervals (CI). Ineligible data for synthesis were described comprehensively.

RESULTS AND DISCUSSION

Selection: Systematic literature search from different databases yielded a total of 66 potential records (break-up shown in Figure 1). After excluding 13 duplicate articles from the search, a total of 53 remained before full-text analysis. A further total of 43 study articles were removed that did not meet the selection criteria. A total

Figure 2. Forest plots showing overall effect of prediabetes on periimplant parameters including (A) plaque index, (B) bleeding on probing, (C) probing depth and (D) crestal bone level.



(A) Plaque index

Study	Non-diabetes Mean	SD	Total	Prediabetes Mean	SD	Total	Mean Difference IV, Random, 95% CI
Alrabiah et al.[22]	24.4	4.7	40	46.7	4.4	39	4.84 [3.96, 5.73]
Abduljabbar et al.[24]	19.2	1.5	42	35.5	4.5	45	4.75 [3.91, 5.58]
Al Amri et al.[20]	23.2	2.5	12	27.3	7.7	12	0.69 [-0.15, 1.53]
Total			**94**			**96**	**3.42 [0.67, 6.17]**

Test for heterogeneity: Chi² = 65.26; df = 2; (P ≤ 0.0001); I² = 96.94%
Test for overall effect: Z = 2.45; (P = 0.015)

(B) Bleeding on probing

Study	Non-diabetes Mean	SD	Total	Prediabetes Mean	SD	Total	Mean Difference IV, Random, 95% CI
Alrabiah et al.[22]	22.6	2.4	40	48.2	3.7	39	8.15 [6.78, 9.51]
Abduljabbar et al.[24]	15.2	0.8	42	36.4	4.1	45	7.00 [5.86, 8.13]
Al Amri et al.[20]	11.2	0.4	12	20.1	2.5	12	4.80 [3.14, 6.45]
Total			**94**			**96**	**6.69 [4.94, 8.45]**

Test for heterogeneity: Chi² = 10.28; df = 2; (P = 0.0058); I² = 80.55%
Test for overall effect: Z = 7.52; (P < 0.001)

(C) Probing depth

Study	Non-diabetes Mean	SD	Total	Prediabetes Mean	SD	Total	Mean Difference IV, Random, 95% CI
Alrabiah et al.[22]	2.2	0.3	40	4.6	0.2	39	9.29 [7.76, 10.83]
Abduljabbar et al.[24]	2.1	0.1	42	4.0	0.4	45	6.36 [5.31, 7.40]
Total			**82**			**84**	**7.77 [4.87, 10.67]**

Test for heterogeneity: Chi² = 9.86; df = 1; (P = 0.0017); I² = 89.86%
Test for overall effect: Z = 5.29; (P < 0.001)

(D) Crestal bone level

Study	Non-diabetes Mean	SD	Total	Prediabetes Mean	SD	Total	Mean Difference IV, Random, 95% CI
Alrabiah et al.[22]	2.3	0.1	40	5.2	0.4	39	9.90 [8.27, 11.52]
Abduljabbar et al.[24]	1.8	0.2	42	3.4	0.6	45	3.93 [3.20, 4.66]
Total			**82**			**84**	**6.87 [0.98, 12.77]**

Test for heterogeneity: Chi² = 44.31; df = 1; (P < 0.0001); I² = 97.74%
Test for overall effect: Z = 2.30; (P = 0.023)

of ten studies were included for full-text reading out of which 4 were excluded subsequently (reasons for exclusion described in Figure 1). Finally, 6 articles were included and processed for tabulation of data, (Abduljabbar, Al-Sahaly, Al-Kathami, Afzal, & Vohra, 2017; Al-Sowygh, Ghani, Sergis, Vohra, & Akram, 2018; Al Amri, Abduljabbar, Al-Kheraif, Romanos, & Javed, 2017; Alrabiah et al., 2018; Alrabiah et al., 2019; Mokeem et al., 2019).

General description of the included studies: Table 1 describes the general features of the included studies. Out of a total of six studies, three studies were retrospective,(Al-Sowygh et al., 2018; Alrabiah et al., 2019; Mokeem et al., 2019). Two studies were case-control,(Abduljabbar et al., 2017; Alrabiah et al., 2018), while one study had a prospective design, (Al Amri et al., 2017). The level of evidence according to CEMB showed four studies had level '2b'(Al-Sowygh et al., 2018; Al Amri et al., 2017; Alrabiah et al., 2019; Mokeem et al., 2019) and two studies had level 3b, (Abduljabbar et al., 2017; Alrabiah et al., 2018). A total of 173 patients with prediabetes and 175 non-diabetic individuals were included in the studies. The mean age of the patients ranged from 43.3 years to 54.3 years. Percentage of male patients was higher than the females.

The total number of implants placed in prediabetic patients were 236, while a total of 235 dental implants were studied in non-diabetic subjects. Mean HbA1c levels in the included data ranged from 6.0% to 6.7%. All investigations related to glycemic status were investigated in serum using HbA1c analyser kits. The overall duration of prediabetic state ranged from 1.9 years to 10.7 years. Only two studies adjusted covariates including periodontal diseases, HbA1c range, total cholesterol, triglycerides, and body mass index, (Abduljabbar et al., 2017; Mokeem et al., 2019). All studies estimated peri-implant parameters by recording peri-implant PI, BOP, PD and CBL. Two studies, in addition to these parameters, estimated peri-implant crevicular fluid levels of AGEs among their patients, (Al-Sowygh et al., 2018; Alrabiah et al., 2018).

Clinical data: Data reporting all clinical peri-implant parameters are shown in Table 2. Some studies reported data based on overall mean with standard deviations (SD), (Abduljabbar et al., 2017; Al Amri et al., 2017; Alrabiah et al., 2019). However, other studies reported data as means with range, (Al-Sowygh et al., 2018; Alrabiah et al., 2018; Mokeem et al., 2019). Plaque index and BOP were reported in percentage mean that ranged from 21.6% to 46.7% and 18.2% to 48.2% in patients with prediabetes, while PI and BOP ranged from 10.6% to 24.4% and 10.8% to 22.6% in patients with prediabetes, respectively. Probing depths ranged from 4.6 mm to 2.2 mm in the prediabetes, while they ranged from 1.3 mm to 2.7 mm in the non-diabetic group, respectively. Crestal bone levels in the prediabetic and non-diabetic ranged from 5.3 mm 1.7 mm and 2.3 mm to 0.7 mm, respectively. Only one study did not report CBL,(Al Amri et al., 2017).

Quality and evidence profile according to GRADE: Quality assessment of the studies is presented in Table 3 according to ROBINS-I tool,(Sterne et al., 2016). An overall quality assessment showed low to moderate risk of bias, which in the majority of the studies originated from the presence of bias and other covariates in the studies and bias in selection of the reported outcomes. Table 4 demonstrates an overall summary of the various factors used to rate the quality of evidence and strength of recommendations according to GRADE,(Guyatt et al., 2011). Altogether, the strength of approval based on the body of the evidence developing from this study is characterized to be moderate. Given that the effect is large for prediabetic state, the direction of recommendation and suggestions emerging from this meta-analysis is strong in favour of prediabetes in the deterioration of peri-implant health.

Final outcomes and meta-analysis: Based on the qualitative assessment and final conclusions described in the included studies, it was observed that patients with prediabetes show worse clinical and radiographic peri-implant parameters compared with non-diabetic subjects, (Abduljabbar et al., 2017; Al-Sowygh et al., 2018; Al Amri et al., 2017; Alrabiah et al., 2018; Alrabiah et al., 2019; Mokeem et al., 2019). Quantitative data in the form of meta-analyses for each variable was conducted. Only those studies presenting data in the form of overall means and SD were included in the meta-analysis. A total of three studies for PI, BOP and PD reported data in range values and not SD,(Al-Sowygh et al., 2018; Alrabiah et al., 2018; Mokeem et al., 2019). In addition, one study reported their outcomes of PD in percentage of ≥4mm(Al Amri et al., 2017). (Table

Table 1. Description of the included studies in chronological order

Author et al; Year; Journal name	Study design/ Level of evidence*	Demographics (Total number of patients/implants; mean age in years; male/female ratio; mean HbA1c levels)	Investigation of glycemic status – duration of prediabetes	Confounding factors adjusted (Yes/No)	Peri-implant evaluation	Final outcome
Alrabiah et al.22, 2019; Clinical Implant Dentistry and Related Research	Retrospective /2b	Prediabetes: 39/78; 54.3; 39/0; 6.1% Non-diabetes: 40/ 80; 51.2; 40/0; 4.1%	Serum HbA1c analysis – 5.4 years	No	Plaque index Bleeding on probing Probing depth Crestal bone loss	Peri-implant tissue inflammation and crestal bone loss are worse around dental implants in prediabetic patients compared with non-diabetic individuals.
Alrabiah et al.23, 2018; Clinical Implant Dentistry and Related Research	Case-control/3b	Prediabetes: 30/42; 52.5; 14/16; 6.1% Non-diabetes: 30/39; 54.1; 15/15; 4.7%	Serum HbA1c analysis – 2.7 years	No	Peri-implant crevicular AGE levels Plaque index Bleeding on probing Probing depth Crestal bone loss	Clinical and radiographic peri-implant parameters were worse and levels of AGEs were increased in patients with prediabetes compared with non-diabetic individuals.
Abduljabbar et al.24, 2017; Acta Odontologica Scandinavica	Case-control/3b	Prediabetes: 45/45; 53.4; NA; 6.1% Non-diabetes: 42/42; 51.1; NA; 4.5%	Serum HbA1c analysis – 1.9 years	Yes (periodontal disease)	Plaque index Bleeding on probing Probing depth Crestal bone loss Number of missing teeth	Periodontal and peri-implant parameters were worse among patients with prediabetes compared with non-diabetic controls.
Mokeem et al.25, 2019; Clinical Implant Dentistry and Related Research	Retrospective /2b	Prediabetes: 22/35; 51.4; 13/9; 6.0% Non-diabetes: 25/32; 46.2; 17/8; 4.6%	Serum HbA1c analysis – 3.1 years	Yes (HbA1c, total cholesterol, triglycerides, body mass index)	Plaque index Bleeding on probing Probing depth Crestal bone loss	Clinical and radiographic peri-implant parameters were worse in patients with prediabetes compared with non-diabetic individuals.
Al Amri et al.26, 2017; Clinical Oral Implants Research	Prospective /2b	Prediabetes: 12/NA; 44.5; 12/0; 6.1% Non-diabetes: 12/ NA; 43.3; 12/0; 4.4%	Serum HbA1c analysis – NA	No	Plaque index Bleeding on probing Probing depth HbA1c	Dental implants inserted in prediabetic and healthy patients have similar success rates and remain clinically and radiographically stable after 1-year follow-up.

Al-Sowygh et al.27, 2018; Clinical Implant Dentistry and Related Research	Retrospective /2b	Prediabetes: 25/36; 51.5; 13/12; 6.7% Non-diabetes: 26/42; 50.1; 13/13; 5.8%	Serum HbA1c analysis – 10.7 years	No	Peri-implant crevicular AGE levels Plaque index Bleeding on probing Probing depth Crestal bone loss	Clinical and radiographic peri-implant parameters were worse and levels of AGEs were increased in patients with prediabetes compared with non-diabetic individuals

AGE; advanced glycation end-products, HbA1c; glycated haemoglobin A1c, NA; not available
* Level of evidence of the included studies estimated using Oxford Centre for Evidence-Based Medicine (CEBM).

Table 2. Clinical and radiographic peri-implant data of the included studies.

Author et al.	Plaque index (%)	Bleeding on probing (%)	Probing depth (mm)	Crestal bone levels (mm)
Alrabiah et al.22	Prediabetes: 46.7 ± 4.4 Non-diabetes: 24.4 ± 4.7	Prediabetes: 48.2 ± 3.7 Non-diabetes: 22.6 ± 2.4	Prediabetes: 4.6 ± 0.2 Non-diabetes: 2.2 ± 0.3	Prediabetes: Mesial – 5.2 ± 0.4 Distal – 5.3 ± 0.2 Non-diabetes: Mesial – 2.3 ± 0.1 Distal – 2.3 ± 0.1
Alrabiah et al.23	Prediabetes: 22.3 (16.2-25.9)	Prediabetes: 24.7 (16.1-29.8)	Prediabetes: 2.7 (2.1-3.5)	Prediabetes: 2.1 (1.3-3.0)
	Non-diabetes: 10.6 (6.4-14.8)	Non-diabetes: 13.6 (5.5-15.2)	Non-diabetes: 1.3 (0.8-1.9)	Non-diabetes: 0.7 (0-1.2)
Abduljabbar et al.24	Prediabetes: 35.5 ± 4.5	Prediabetes: 36.4 ± 4.1	Prediabetes: 4.0 ± 0.4	Prediabetes: 3.4 ± 0.6
	Non-diabetes: 19.2 ± 1.5	Non-diabetes: 15.2 ± 0.8	Non-diabetes: 2.1 ± 0.1	Non-diabetes: 1.6 ± 0.2
Mokeem et al.25	Prediabetes: 24.6 (19.3-29.6)	Prediabetes: 24.7 (16.1-29.8)	Prediabetes: 2.2 (2.0-3.1)	Prediabetes: 1.9 (1.1-2.8)
	Non-diabetes: 11.4 (6.4-14.8)	Non-diabetes: 13.6 (5.5-15.2)	Non-diabetes: 1.8 (0.7-2.1)	Non-diabetes: 0.8 (0-1.3)
Al Amri et al.26	Prediabetes: 27.3 ± 7.7	Prediabetes: 20.1 ± 2.5	Prediabetes: 5.1 ± 1.4†	
	Non-diabetes: 23.2 ± 2.5	Non-diabetes: 11.2 ± 0.4	Non-diabetes: 2.7 ± 0.5†	NA
Al-Sowygh et al.27	Prediabetes: 21.6 (14.5-24.7) Non-diabetes: 12.3 (7.6-15.9)	Prediabetes: 18.2 (11.4-26.7) Non-diabetes: 10.8 (6.0-13.1)	Prediabetes: 2.6 (2.0-2.9) Non-diabetes: 1.4 (0.7-2.1)	Prediabetes: 1.7 (1.5-3.1) Non-diabetes: 0.8 (0-1.1)

Data represented in percentage of ≥4mm, NA; not available

2). Therefore, these studies were not considered for meta-analysis and excluded.

Significant heterogeneity was observed for all the parameters including PI, BOP, PD and CBL, therefore a random effect model was used. Considering the effects of prediabetes, significant heterogeneity for PI (χ^2=65.26, P<0.0001, I2 =96.94%), BOP (χ^2=10.28, P=0.0058, I2 =80.55%), PD (χ^2=9.86, P=0.0017, I2 =89.86%) and CBL (χ^2=44.31, P<0.0001, I2 =97.74%) was noticed between both the groups. The overall weighted mean difference for PI (SMD=3.42, 95% CI= 0.67 to 6.17, P=0.015, Figure 2A), BOP (SMD=6.69, 95% CI= 4.94 to 8.45, P<0.001, Figure 2B), PD (SMD=7.77, 95% CI= 4.87 to 10.67, P<0.001, Figure 2C) and CBL (SMD=6.87, 95% CI= 0.98 to 12.77, P=0.023, Figure 2D) showed statistically significant differences between prediabetes and non-diabetic groups, respectively.

The present systematic review and meta-analysis was based on the hypothesis that no significant differences are observed in implant survival rate between patients with prediabetes and those who are non-diabetic and no significant differences are observed between these groups with regards to clinical and radiographic peri-implant parameters including peri-implant clinical and radiographic status around dental implants. The null hypothesis was rejected, and all clinical studies showed worse peri-implant inflammatory parameters around dental implants placed in patients with prediabetes compared with non-diabetic controls. It is well-known that constant hyperglycemia and elevated blood glucose levels lead to non-enzymatic glycosylation of several serum proteins that subsequently leads to the formation and accumulation of AGEs in the body tissues, (Katz et al., 2005; Joseph Katz, Yoon, Mao, Lamont, & Caudle, 2007). This constant build up also leads in the production of several proinflammatory cytokines that are responsible in the destruction of soft and hard tissues of periodontal and peri-implant structures, (Pertyska-Marczewska et al., 2004 and Akram, Alqahtani, Alqahtani, Al-Kheraif, & Javed, 2019). There is impairment in the blood glucose levels in prediabetic state which may consequently lead to the similar proinflammatory destructive pathways. In the present systematic review, it was observed that scores of PD, CBL and peri-implant inflammation were higher in patients with prediabetes compared with non-diabetic individuals.

The reason for these increased scores may be associated with raised levels of AGEs as described in the two clinical studies, (Al-Sowygh et al., 2018; Alrabiah et al., 2018). Nevertheless, the outcomes of this meta-analysis should be interpreted with caution due to several important factors that may not be found in the included studies and these factors have a profound effect on peri-implant health. For instance, it is well-known that cement retained prostheses showed less CBL as compared to screw-retained, (Lemos, de Souza Batista, et al., 2016). Short dental implants have generally higher risk of failure as proved in a recent meta-analysis, (Lemos, Ferro-Alves, Okamoto, Mendonça, & Pellizzer, 2016).

The included studies also described a variation in the mean duration of implants in service. These important factors, yet significant should be considered while giving a definitive conclusion. Recent data suggests that obesity have a significant impact on the overall survival of dental implants, (Alkhudhairy, Vohra, Al-Kheraif, & Akram, 2018 and Alasqah et al., 2019;). These studies demonstrate that chronic systemic

Table 3. Risk of bias in non-randomised studies - of interventions (ROBINS-I) tool

Author et al.	Bias due to confounding	Bias in selection of participants into the study	Bias due to missing data	Bias in measurement of outcomes	Bias in selection result of the reported	Overall bias
Alrabiah et al.22	Moderate	Low	Low	Low	Moderate	Moderate
Alrabiah et al.23	Moderate	Low	Low	Low	Moderate	Moderate
Abduljabbar et al.24	Low	Low	Low	Low	Moderate	Low
Mokeem et al.25	Low	Low	Low	Low	Moderate	Low
Al Amri et al.26	Serious	Low	Moderate	Low	Moderate	Moderate
Al-Sowygh et al.27	Moderate	Low	Low	Low	Moderate	Moderate

Table 4. Summary of findings table on body of the estimated evidence profile (GRADE, 2015) and appraisal of the strength of the recommendation regarding the impact of prediabetes on clinical peri-implant parameters.

Determinants of quality	Prediabetes
Study design Number of studies	Cross- sectional nature
n = 6 (Figure 1) Comparison n = 6	6
Risk of bias	Moderate
Consistency (Figure 2 and 3) Directness	Consistent Generalizable
Precision	Rather not
Publication bias	precise Not for a
(Appendices S1 and S2)	PDT but for LI
Magnitude of the effect	Large
Strength of the recommendation based on the body of evidence	Moderate
Direction of recommendation	Strong in favour of prediabetes

inflammation as seen in obesity may show worse peri-implant inflammatory scores compared to non-obese counterparts. In a recent case-control investigation by Vohra et al. revealed that elevated levels of serum c-reactive proteins may lead to worse peri-implant inflammation in different categories of obese individuals depending on their body mass index (BMI), (Vohra, Alkhudhairy, Al-Kheraif, Akram, & Javed, 2018). It is noted that some of the studies did not report the overall anthropometric assessments including BMI, waist circumference, or even total fat mass. This important missing parameter may have also skewed the results. The present meta-analysis contains some important limitations that should not be overlooked.

For instance, most of the study designs were retrospective. Their questionnaire data (as described in their own studies) reports assessments that relied on recall abilities of the patients. For this reason, due to the inclusion of retrospective cohort data in this meta-analysis does not solely determine causation. Furthermore, the inclusion of studies from the same author groups, (Alrabiah et al., 2018; Alrabiah et al., 2019) and Abduljabbar et al., (2017) may have produced significant bias. Although calibration was performed for clinical measurements, but accuracy of measurements was not calibrated in radiographic assessments which might contribute to geometric errors on the conventional radiographs in the included studies. Moreover, the limited number of studies included does not actually help to translate the impact of prediabetes on peri-implant health. Further well-designed prospective studies with well-designed methods and control of systemic and other local factors should be undertaken in order to establish better understanding and strong conclusions regarding prediabetes and peri-implant inflammation. Within the limitations and the direction of recommendation emerging from this meta-analysis that proves to be strong in favour of prediabetes in the deterioration of peri-implant health compared to non-diabetic patients.

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An Updated Review on the Spiders of Order Araneae from the Districts of Western Ghats of India

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ABSTRACT

Spiders belonging to order araneae are one of the biggest groups of predaceous organisms in the animal kingdom. Along with their diverse structure, they are popular for their tough silk and spider venom which is of pharmaceutical importance.Also they are playing a major role in lower food web in the ecosystem to maintain the ecological equilibrium and are one of the best bioindicators of natural ecosysytems.Now a days, due to the habitat destruction caused by natural calamities and anthropogenic activities ,the number of spiders species are found to be declined, as a result number of species of spiders are getting extinct before they are explored. One of the major hotspots of biodiversity of India is Western Ghats, known for their high species diversity. Taking into account the importance of spiders as bioindicators of environment, the present article was aimed to review on Araneae diversity from Satara Sangli and Kolhapur districts lying in the Northern Western Ghat regions of Maharashtra, India . This study shall help to record the number of species found in this regions. This review provides a checklist of total 27 families of 101 genera representing 178 species along with some new species recorded with special reference to their behaviour, habitat and variations among them. The review mainly provides a baseline information for the future study on diversity of spiders.From this review it is concluded that Northern Western Ghats being one of the biggest hotspots of diversity have a large number of spiders species, which are very less as compared to the spider diversity of India as suggested by the world catalogue of spiders, suggesting for an urgent need to explore further the areneae diversity in order to maintain the ecological equilibrium and the products obtained from them.

KEY WORDS: ARACHNIDA, ARANEAE, SPIDER, BIODIVERSITY, WESTERN GHATS.

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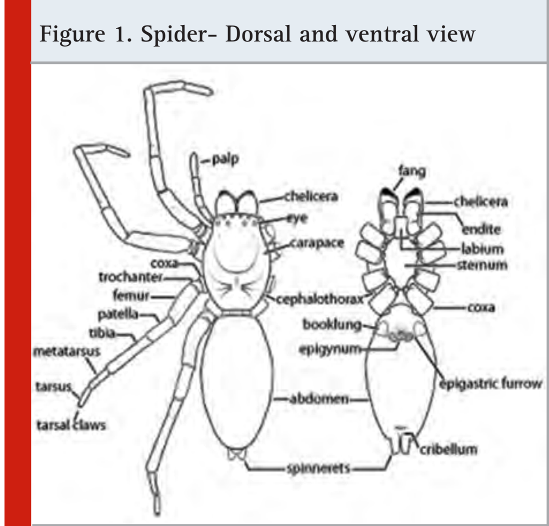
INTRODUCTION

Spiders are the ancient Arthropods belonging to the class Arachnida having history of about 350 million years ago. These are the air breathing arthropods known to have special characteristics containing eight legs, two to eight eyes and spinnerets to spin the silk.They differ from class Insecta due to the absence of antennae and wings. Class Arachnida contains orders- Scorpiones, Schizomida, Amblypygi, Uropygi, Opiliones, Pseudoscorpiones, Palpigradi,Solifuge, Ricinulei, Acari and Araneae, (Tikader,1982; Sebastian and Peter 2009). Spiders belonging to order Araneae differ from other orders of Arachnids by the presence of pedicel that joins the cephalothorax and the abdomen. Their special characteristic is the presence of spinnerets which secrete silk, this character differs them from the mites, which also secrete the silk by the glands in their palp as there are no spinnerets. Order Araneae constitutes the largest order, which ranks 7th in all over diversity of species. Spiders are very small to large sized arthropods with some special features within environment and ubiquitous in terrestrial habitat, (Turnbull,1973;Preston ,1984; Nyfeller,1987).

Arachnids are an important but generally poorly studied group of phylum Arthropoda. Many authors are making a lot of efforts to bring forward the research on spider biology and educate its importance to society. Many reviews have been collected from international context which have helped in supporting research on spiders, which are meant to have precious role in ecology as they are mostly predatory in nature which help in reducing insect pests and invertebrate population in most ecosystems, (Jennings,1986; Wise, 1993; Russell 1999;Maloney,2003 and Plantnick 2019). Many researchers have conducted baseline studies involving role of spiders as biological, ecological indicators of natural ecosystem (McIver,1990;Churchill,1997;Maelfait, 1998) and in conservation planning, (Kremen ,1993). Allred (1969) and Allred (1976) also reported diversity of spiders in Arizona and Utah after new power plant installations in Nevada at the Nevada Nuclear test site to indicate any harmful changes in the ecosystem due to this plant.

Figure 1. Spider- Dorsal and ventral view



Wise (1993) recorded importance of spiders in ecological web. Spiders are widely distributed group of predaceous organisms in the animal kingdom, (Riechert, 1984). Knowing the importance of presence of diverse Araneo fauna and its role in the ecosystem, the present review was aimed on the spider diversity of Satara, Sangli, Kolhapur districts which are the important locations of Western Ghats of Maharashtra known for their varied and rich biodiversity. According to World Spider Catalogue (Version 20.5) by Plantnick (2019), there are 48,334 species belonging to 4,143 genus representing 120 families. The pioneer contribution in Arachnology of Indian Spiders was given by European arachnologist, Stoliczka (1869).

Earliest contribution in arachnology was given by Blackwell (1867), Karsh (1873),Simon (1887), Thorell (1895), Pocock (1900),Malhotra (1980) and Tikader (1980, 1982). Gajbe (1978-2009) gave precious contributions on the Spiders of India. According to Indian Spiders checklist given by Siliwal (2005), 1442 species belonging to 361 genus of 59 families have been recorded and in 2012, Keswani recorded 1685 species of 438 genus belonging to 60 families, out of them, 91 species are of Infraorder Mygalomorphae and 1595 species of Araneomorphae. As per Tikader (1974), knowledge on diversity and distribution of spiders in Maharashtra is sparse, as compared to other regions of the world. Gazetteer of India which

included 90 species, 14 families. Hippargi (2011b) recorded 19 families from Lonar, 25 families from Melghat and 31 families from Southern Tropical thorn forest, Solapur. From Western Ghats, Sebastian (2012) recorded 275 species belonging 139 genus and 39 families. Wasankar (2016) recorded 37 species of spiders belonging to 25 genera representing 17 families from Buldhana. 21 species representing 19 genus of 13 families recorded by Gajbe (2016) from Nagpur. 71 species of 15 families by Maheshwari (2018), and 104 species of 18 Families by Deshmukh (2014) recorded from Satpura ranges. From Amravati 31 species of 8 genera of 1 family were recorded by Deshmukh (2018). From Vidarbha in cotton fields 70 species belonging to 29 genera of 7 families were recorded, and in orange fields total 64 species of 13 families were recorded by Deshmukh (2017). Gajbe (2016) recorded 13 species of 9 families from Chandrapur and from Akola 26 species were recorded by Shirbhate (2017) (Table No 2).

Looking at the scenario of diversity of spiders from Maharashtra, a data from 2012-2019 was compared with India (Keswani,2012) which suggests that a lot of species still have to be explored on large scale before any natural calamities or any other environmental hazards make any species extinct without being explored Fig No.4. So this review has been carried out on to present the current status of spider diversity in three regions of Maharashtra, Satara, Sangli and Kolhapur which are the main parts of Northern Western Ghats known for one of the hottest biodiversity spots of India, [Fig No.2]. Along with their importance as bioindicators they are also known popularly for Spider venom, which plays precious role in pharmacological extracts, by preying larva and adults of mosquito which carry malarial parasite (Choi, 2004; Ndava,2018). Spiders contain venom which is rich in proteins, containing disulfide peptides which have affinity and specificity towards specific subtype of ion channels and receptors. According to a report by Honor,(2015) only 0.01 % of its enormous pharmacological component has been explored till now. This discovery has accelerated the hope of development of novel molecules from such animal sources for drug discovery. This has also been innovatively used in medical,pharmaceuticals, and therapeutics, as a non polluting biopesticide, (Bode, 2001; Fry, 2009; Saez,2010; Herzig,2011;Windley, 2012; King and Hardy 2013; Mammola,2017; Li, 2017; Pineda,2018; Halarhkar,2018 and Luddecke,2019)

Recently various non chemical control methods are used in integrated pest management. Spiders in the rice field control the population of plant hoppers and leafhoppers, also in the cotton fields. And it is observed that web weavers controls the population of adult white fly and hunters control the population of nymph of pests in the field, without damaging plants. Thus they maintain the equilibrium in the ecosystem by playing major role in lower food web in the ecosystem, (Hamamura,1969; Sasaba,1973; Gavarra,1973; Samal, 1975; Kobayashi,1977; Chiu,1979;Vanden,1982; Holt,1987; Tanaka,1989; Jeyaparvathi, 2013; Lee;

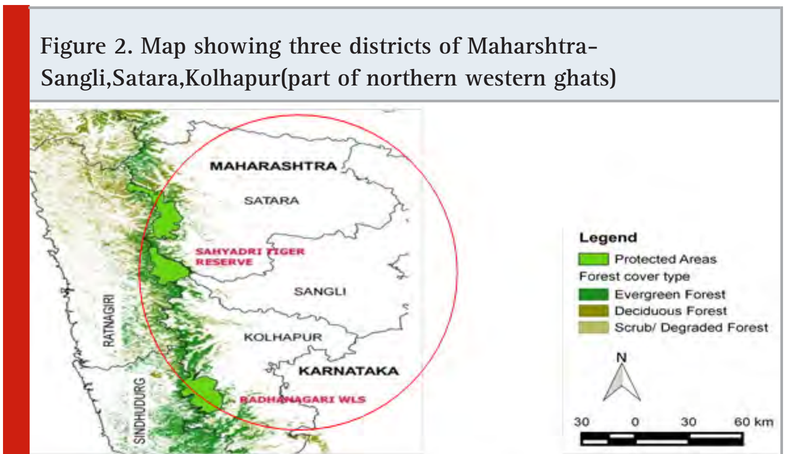


Figure 2. Map showing three districts of Maharshtra- Sangli,Satara,Kolhapur(part of northern western ghats)

Table 1. List of Family wise Distribution of Spiders

Sr.No	Name of Family	Genus
01	Araneidae	*i)Araneus ii)Argiope iii) Cyclosa iv)Gasteracantha v)Eriovixia vi)Larinia vii)Neoscona ix) Nephila x)Parawixia xi) Plebs xii)Guizygiella xiii) Cyrtophora xiv)Arachnura xv) Telecantha xvi)Poltys*
02	Salticidae	*i)Aemonea ii) Bavia iii)Brettus iv) Currhotus v)Epeus vi)Epocilla vii) Hasarius viii) Hyllus ix)Menemerus x) Phintella xi)Marpissa xii)Myrmarachne xiii) Plexippus xiv)Portia xv) Rhene xvi)Telamonia xvii)Thiania*
03	Lycosidae	*i)Geolycosas ii)Hippasa iii) Lycosa iv)Pardosa v) Archtosa vi)Evippa*
04	Thomisidae	*i)Amyciaea ii)Misumena iii)Oxytate iv) Ozyptila v)Runcinia vi) Thomisus vii)Xysticus viii)Tmarus*
05	Sparassidae	*i)Heteropoda ii)Olios*
06	*Theridiidae*	*i)Achaearanea ii)Argyodes iii) Chikunia iv)Enoplognatha v)Episinus vi) Euryopis vii)Nesticodes viii) Phoroncidia ix)Phycosoma x) Rhomphaea xi)Steatoda xii)Theridion xiii)Theridula xiv)Latrodectus xv)Propostira*
07	Oxyopidae	*i)Oxyopes ii)Peucetia*
08	Pholcidae	*i)Artema ii)Crossopriza iii)Pholcus*
09	Pisauridae	*i)Pisaura ii)Nilus iii)Thalassius iv)Dolomedes v)Perenethis*
10	Tetragnathidae	*i)Leucauge ii)Opadometa iii)Tetragnatha*
11	Gnaphosidae	*i)Gnaphosa ii) Scotophaesis iii) Poecilochroa iv)Scopoides v)Zelotes*
12	Eresidae	*i)Stegodyphus*
13	Corinnidae	*i)Castianeira*
14	Hersilidae	*i)Hersilia*
15	Philodromidae	*i)Philodromus ii)Tibellus iii)Tibilus*
16	Ctenidae	*i)Ctenus*
17	Dipluridae	*i)Diplura*
18	Linyphiidae	*i)Neriene*
19	Euticharidae	*i)Chieracanthium*
20	Sicariidae	*i)Loxosceles*
21	Ulboridae	*i)Uloborus Zosis*
22	Zodariidae	*i)Mallinella ii)Zodarion*
23	Miturgidae	*i)Cheiracanthium*
24	Scytodidae	*i)Scytodes*
25	Clubionidae	*i)Clubiona*
26	Theraphosidae	*ii)Unknown*
27	Nephilidae	*iii)Nephila*
Total	27	101

Found species of spiders in study area –

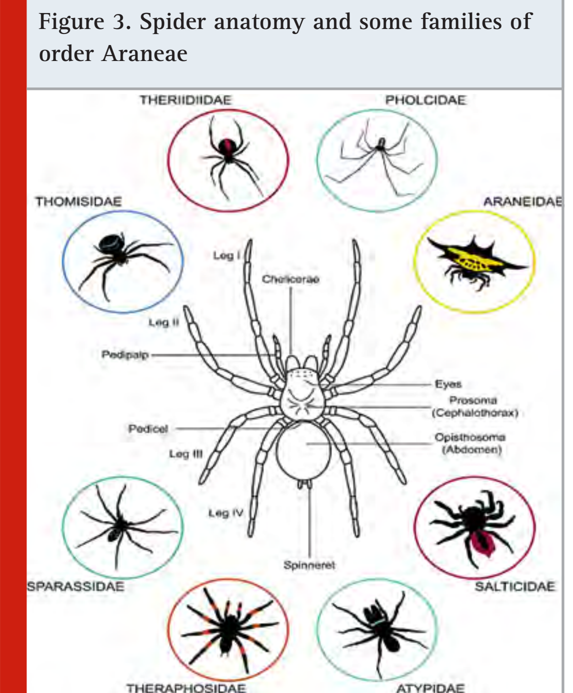
o *Araneus mitificus* , *Stegodyphus sarasinorum* ,*Hyllussemicupreus*

o *Argiope aemula* , *Argiope anasuja* ,*Castianeira zetes*

o *Cyclosa confrag* , *Neoscona bengalensis* , *Scytodes fusca*

Cotes, 2018; Tabasum,2018 and Ndava,2018). Spiders silk is used to explore evolution, biomaterial engineering, to check physical properties of protein fibres (Hinman,2000; Heim, 2009; Mammola,2017).Spiders are very careful about habitat changes. Their occurrence depends on the microclimatic characters,vegetation of biotic and abiotic factors. Their diversity is going to decrease due to habitat destruction, anthropogenic activities, environmental changes, cannibalism, territoriality , natural calamities hence they are on the verge of extinction, (Pinkus, 2006; Cardosa, 2010; Lee;Cardosa,2017;Tabasum,2018; Halarnkar,2018). Taking into consideration the importance of spiders as biocontrol agents ,bioindicators of ecosystem and therapeutic potential of Venom and economic imporatance of sil produced by them ,it is a matter of concern to protect the diversity of spiders found in such area of varied biodiversity and pave the way to explore their pharmaceutical potential. The species level spider list used here is taken from literature, based on i) modern literature ii) new species records with genitalia dissection and morphological, behavioural characters. All the valid Genus and species are summarised. The record of spiders of Satara, Sangli, Kolhapur has not yet been listed before so it was short of references. Total 27 families representing 101 genera belonging to 178 species have been recorded by authors in a diversity of papers. Some new species of spiders have been also recorded from Satara district. These families are present in every habitat. Infra-order Araneomorphae shows its dominance over Mygalomorphae They show different types of web pattern. New species are recorded according to morphological and reproductive characters also the notes of its life stages, keys of identification, and their behavior were studied. The organisms morphology, habit, habitat, its life cycle study is crucial for its study and identification. More, (2010) recorded dominant families are Oxyopidae, Thomisidae, Araneidae, and Salticidae from the Radhanagari wildlife sanctuary, Chandoli national park, Koyana wildlife sanctuary.

Figure 3. Spider anatomy and some families of order Araneae



Bendre, investigated total 11 species belonging to 6 families .Kulkarni, (2011) studied Ecology and behaviour of Argiope species during September to November 2010 .Kulkarni, (2011) recorded new species of spider belonging to Family, Oxyopidae-Oxyopes sataricus.Kulkarni,(2012) recorded the spider Arachnura angora from Maharashtra . Kulkarni , (2012) recorded new flower spider

Figure 4. Comparison of species wise diversity of spiders of year 2012 (Keswani) from India with three districts of Maharashtra till 2019

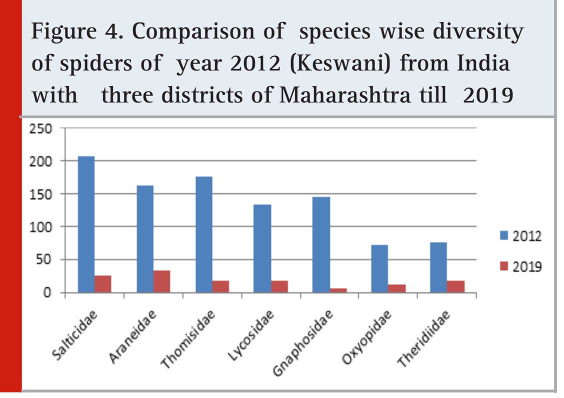


Table 2. Diversity of Spiders in Maharashtra from some areas-

Areas in Maharashtra	Study Year	Family	Genus	Species
Buldhana,Wasankar, (2016).	2014-2015	17	25	37
Nagpur,Gajbe,(2016).	2014-2015	13	19	21
Satpura Ranges, Deshmukh,	2012-2013	18	-	104
(2014), Maheshwari , (2018).	2014-2015	15	-	71
Amravati,Deshmukh,(2018).	2017-2018	1	8	31
Vidarbha (Cotton fields and orange	2011-2012	7	29	70
fields respectively),				
Deshmukh,(2017).		13	32	64
Chandrapur, Pawan ,Gajbe,(2016).	2012	9	12	13
Akola, ,Shirbhate, (2017).	2015-2016	1	11	26

Thomisus sikkimensis with note on its courtship behaviour. Karanjkar in his minor project recorded 20 Families representing 40 Genus and 90 species from Satara Tehsil. Mirza ,(2012) recorded a new species of the Mygalomorphae spiders belonging to family Idiopidae (trapdoor spiders). More, (2013) recorded 24 families representing 150 species of 78 Genera from Bamnoli region of Koyana wildlife Sanctuary during the study year 2011- 2013. Kulkarni, (2013) recorded 63 Genus of 25 Families and have given Generic checklist which showing Araneidae as a dominant family. Kulkarni ,(2014) recorded new species Tylorida satarensis, with addition of field observations of that species.Kulkarni ,(2015) described habit and conservation status of male Tylorida satarensis.

Kulkarni ,(2015) recorded Genus Siler Simon,1889 .More (2015) recorded 90 species belonging to 54 genus from Zolambi region of Chandoli National Park. More (2015) recorded 18 families representing 61 species from Vakoba Devrai region of Radhanagari Wildlife Sanctuary. More (2015) investigated 16 families representing 58 species belonging to 38 genus from Rundiv, Sidheshwar and Ramnadi area of Chandoli national park. Lanka,(2015), investigated 18 families during year 2011-2013 and in that most dominant families are Araneidae representing 16species and Salticidae representing 10 species . More(2016) recorded 18 families representing 70species belonging to 48 genus from Dandoba hill forest. Lanka (2017),recorded 106 species belonging to 78 genus of 24 families from Radhanagari wildlife sanctuary. Kulkarni, (2017) reported a new species of spiny spider Meotipa sahyadri belonging to family Theridiidae. Also he have given the phylogenetic analysis of that spider and relationships between Meotipa with Chrysso.Sarwade, (April 2017) studied diversity and seasonal variation of spiders from Mhaishal lake and Brahmanath lake Khanderajuri, from Miraj Tehsil of Sangli. Depending on the habitat ,climatic conditions , altitude and latitude diversity of spiders also changes. .As they are shy creatures, hide from others. These primitive spiders are on the way of extinction and rarely found. They must be documented as they are less examined by the peoples.

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The Reforms of the General Pension Scheme: Evidence From the Tunisian Health Insurance Fund

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ABSTRACT

This paper describes the recent Tunisian reform and available options on major issues within this reform framework A country's economic growth and political stability are two very important factors in the smooth running of an economy. Tunisia stands crippled by a climate of economic uncertainty. This has led the economy to suffer very serious collateral damage. Supply and demand seem to be affected, weakening all macroeconomic balances. The National Pension and Social Security Fund known as the CNRPS is in a critical situation and raises many fundamental and complex questions, which have been the subject of an ongoing hot debate. The objective of this study was to analyse and describe the reform of the general pension scheme and health insurance fund in Tunisia. The reform of the general pension scheme and health insurance fund is among the first in Africa countries. In order to find the best possible evidence, this was a review of online grey literature on regulatory approaches to reform the national pension and Social Security Fund known as the CNRPS and propose solutions related to the general pension system. The whole working career is the basis for the pension and the benefit drawn at a given age. Tunisian health insurance as CNRPS organization should robust retirement income system that delivers good benefits.

KEY WORDS: HEALTH INSURANCE, GENERAL PENSION, REFORM, CONTRIBUTION RATE, RETIREMENT, REVISION of PENSION.

ARTICLE INFORMATION

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INTRODUCTION

Investigating the satisfaction of social security beneficiary and their expectations is of particular importance the rapid economic growth of a country plays a very important role in its national and international development. Some several researches have more or less explicitly – 2004 pointed to other aspects of recent pension restructuring. Privatization (Hinrichs, 2004), the segmentation between contributory and non-contributory benefits (Palier, 2002) and policies addressing new social risks (Bonoli, 2003, 2004) can be mentioned as selective examples. All persons who work in corporate enterprises, including company directors, are considered to be employees (OECD, 2018;2019).

However, Tunisia's economic situation has changed and has thus affected the CNRPS pension fund. Since then, this latter has been facing a threat that still remains serious with regard to the imbalance in the general pension system (Department of Public Expenditure and Reform, 2019). Knowing that the main source of funding for the fund's plan is the pay-as-you-go scheme, which is originally the concatenation of the two words: contribution (by the employee with a rate of 8.2%) and contribution (by the employer with a rate of 12.5%). It is therefore the time to ask the following question: What pension plans does the CNRPS have? What are the causes of the budget deficit of this system? What are their consequences on the cash register? And what must be done to face this deficit in order to help the fund survive?

The Fund's Plans: The CNRPS operates social security schemes for public officials, local authorities, public administrative institutions, and national companies. These are essentially four pension plans to mention: The death benefit plan: This plan is financed through contributions deduced from the salaries of the active insured and retired employees and investments generated by the growth of the plan's reserves. The subsidized scheme: The pensions provided by the latter are financed entirely from the State budget. The general regime: General pension plans are plans whose beneficiaries are employees of large public institutions. In Tunisia, the legal retirement age is still 60 years, but it is expected to be extended to 62 years very soon. The special regime: Special pension schemes are pension schemes from which members of a governorate benefit. Some different pension schemes keep the unequal conditions without any coordination. (As Pension Reform in France). The fund reserves: Reserves are built up from plan surpluses and depend on the length of time and investment rate

Table A.1: Receipts amounts evolution and their growth rate

YEAR	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Receipts	817	881	959	1089	1231	1404	1542	1770	2033	2250	2399
Amount	780	571	416	037	439	820	992	134	110	216	583
Growth rate in (%)		7,80	8,83	13,51	13,08	14,08	9,84	14,72	14,86	10,68	6,64

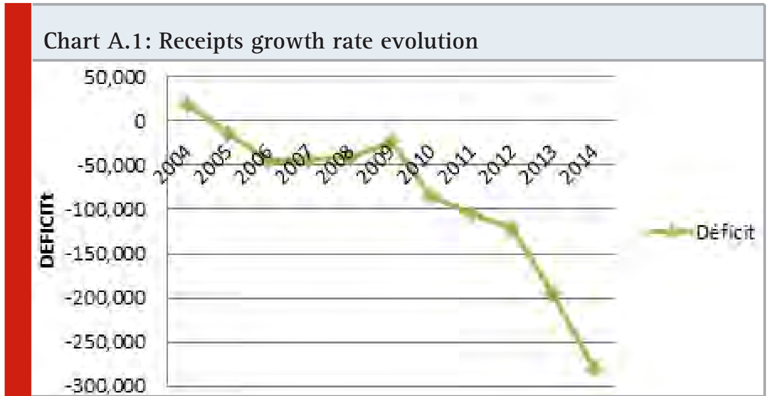


Chart A.1: Receipts growth rate evolution

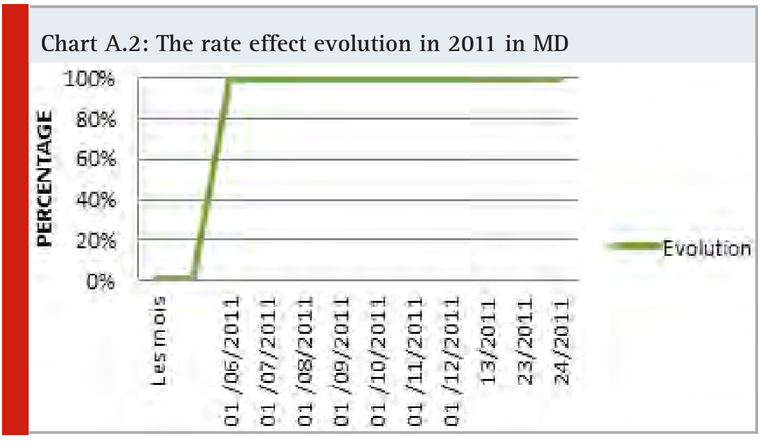
and the size of the gap between the contribution rate required to maintain equilibrium and the current contribution rate. These reserves can be used to cover the annual deficits of a scheme in times of economic difficulties and can be a source of funding for the social security system through the income they generate when they bear fruit. The fund system: Since Tunisia's independence in 1956, social security systems have diversified and social security coverage has continued to expand to cover the largest number of the employed population. In this regard, it is possible to consider two models of pension systems: pay-as-you-go or funded. The pay-as-you-go system: In a pay-as-you-go pension system, the financing of pensions is ensured by compulsory deductions from the working population. These deductions are then redistributed to pensioners in the form of a pension. Working people do not contribute for themselves when they retire, but for today's retirees. Their retirement will be ensured by the working people of tomorrow. The funded system: Unlike the pay-as-you-go system, the funded system bears on the fact that active population institute savings, individual or collective, that are invested on the financial markets. When they reach retirement age, they draw on it to finance their retirement. Pensions are therefore funded by previous savings, not by a redistribution of contributions to retirees. Retirement, whether funded or distributed, is ensured by the income from working people, and of course for everyone.

Retirement pillars: To fully understand the role of the pension system in financing pensions, it is necessary to go through the pillars of the notion of retirement. These are four: The term "pillars" is a common vocabulary in pension financing. The pillars represent the different sources of income

Table A.2: Increase in pension subsidy rate effect in the second half of 2011

MONTH	SUB RET	SUB PS	SUB PS E3 ESTIMED	SUB PS CALCULED	RATE	Evolution
01/06/2011	68956157,468	22548968,204	1477032,769	24026000,973	11,4802555	
01/07/2011	73607660 ,591	22057042,750	1563498,305	23620541,055	12,46502532	5905135,264
01/08/2011	75918724,374	22793818,647	1553373,857	24347192,504	12,47268643	6086798,126
01/09/2011	85307466,920	25737749,772	1599766,934	27337516,706	12,48210916	6834379,176
01/10/2011	81167863,229	24206593,325	1832786,551	26039379,876	12,46847868	6509844,969
01/11/2011	81103025,872	24256616,594	1750136,529	26006753,123	12,47414862	6501688,281
01/12/2011	82210542,663	24608047,676	1728380,666	26336428,342	12,48620984	6584107,086
13/2011	12608407,583	4052569,931	0,000	4052569,931	12,44485134	1013142,483
23/2011	9387177,420	2853759,138	171142,355	3024901,493	12,41320082	576255,373
24/2011	15276000,971	4665906,875	247910,312	4913817,187	12,43513984	1228454,297
TOTAL	41419775,054					

Chart A.2: The rate effect evolution in 2011 in MD



available to pensioners. We distinguish four pillars: Minimum seniority, past contributions-based pensions, individual savings, and work revenues.

Deficit Causes and their Consequences on The Fund the Main Causes Fall into Three Categories: Cyclical causes, which combine circumstances because of the country's economic situation. Indeed, on the one hand, unemployment and poverty are increasing, then the fund has fewer contributions at its disposal. On the other hand, wages also stagnate and so do social contributions deduced from wages and so do contribution revenues, which lead to slower growth and subsequently to a widening deficit. Exogenous structural causes which represent a set of causes caused by external factors related to the CNRPS system. These can be the increase in health expenditure and the deficit of the sickness branch as well as the old age branch which also generate an increase in the deficit of the system in question. Endogenous structural causes, which are a set of causes formed within the fund system without external factors. They manifest themselves in the social contributions levied on salaries resulting in a decrease in revenues, the reimbursement of health care (see free care) and compensation for sick leave, leading to abuse. These in turn imply increased expenses, implying thus that all the causes presented above have generated a huge deficit in the general balance of the fund.

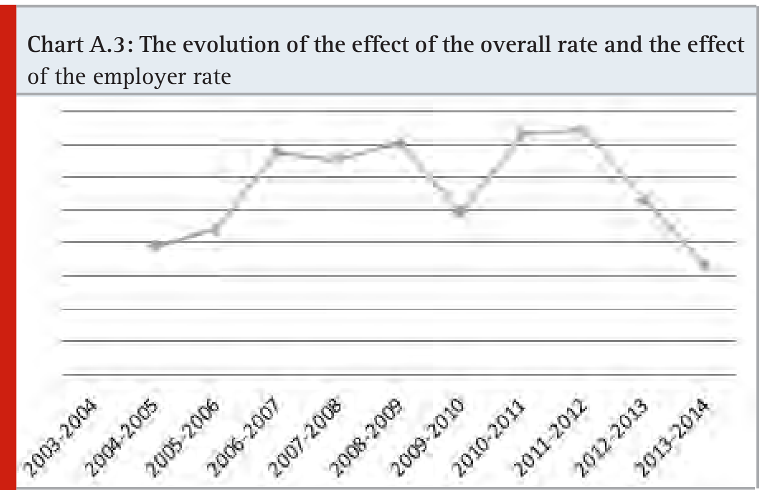
RESULTS AND DISCUSSION

Bearing on the detailed analysis of the deficit of the general pension system, we discuss two main components: revenues and expenditure. We present first the analysis of the evolution of revenues in response to the effect of the growth rate and the effect of the contribution rates. Second, we present the evolution of expenditure in terms of pensions and in terms of the evolution of the demographic component, which is very important. Deficit analysis by revenue: The evolution of the deficit in response to the effect of revenue growth rate: In Table 2.1, the amount of annual revenue has increased. It went from

Table A.3: The evolution of the effect of the overall rate and the effect of the employer rate

YEAR	2003-2004	2004-2005	2005-2006	2006-2007	2007-2008	2008-2009	2009-2010	2010-2011	2011-2012	2012-2013	2013-2014
Rate effect two years by two (%)		7,80	8,83	13,51	13,08	14,08	9,84	14,72	14,86	10,68	6,64
Overall rate effect by four years (%)		13,20		-3,22		-30,14		0,92		-37,84	

Chart A.3: The evolution of the effect of the overall rate and the effect of the employer rate



817780 MD in 2004 to 2399583 MD in 2014. In the graph, revenue growth rate fluctuates from year to year. It is 7.80% in 2005, 14.72% in 2011 and 6.64% in 2014. Table A.2 shows the increase in pension subsidy rate by 1% during the second half of 2011: NB: the increase in the effect of the employer pension subsidy rate increased by 1% compared to the 2nd half of 2011, otherwise employer rate became 12.50% instead of 11.50% for the general scheme. Therefore, bearing on Table A.2, a 1% increase in this rate has a positive and significant effect on the fund's revenues, which is reported in the figure below. In the figure below, we can see that each time the rate changes, whether for the general or special regime, the revenues change gradually. Moreover, the positive effect of an increase in the rate only appears after two years and does not persist because of many demographic factors beyond the control of the fund. These factors are ageing of the population and change in life expectancy of pensioners. Summary Table A.3 and Chart A.3, below, represents the evolution of the overall effect and the effect of the employer rate: In Table A.3 and Figure A.3 above, it can be seen that with each change in the rate, whether for the general or special regime, revenues change gradually and the positive effect of an increase in rates only appears after two years and does not persist because of many demographic factors beyond the control of the fund. These factors are aging of the population and changes in pensioners' life expectancy. The evolution of the deficit by contribution rates:At this level, a distinction should be made between two terms commonly understood by economists, namely contributions deduced from the employee's salary, which is set at 8.20%, and the contribution paid by the employer to CNRPS, which is currently set at 12.5%.So, The income related pension financed by contributions.(here is like The new system for public pensions in Sweden). According to the above table, taken from the CNRPS's control management department, we notice that from July 2011 to 2015, contribution rates for the two agents (employee and employer) are static

Table A.4: Year by year contribution rates evolution

Retirement Effective Date	Affiliated contribution (General scheme)	Employer contribution (General scheme)	Foresight social Affiliated	Foresight social Employer	Death benefit Affiliated	Observations
			-	-	-	
01/10/1985	5%	7%	1%	1%	1%	
01/07/1995	6%	8,20%	1%	1%	1%	
01/07/2002	6,50%	8,70%	1%	1%	1%	
01/07/2003	6,75%	8,95%	1%	1%	1%	
01/07/2004	7%	9,20%	1%	1%	1%	
01/07/2005	7%	9,45%	1%	1%	1%	
01/07/2006	7%	9,70%	1%	1%	1%	
01/01/2007	7,40%	10 ,30%	1%	2%	1%	
01/01/2008	7 ,80%	10,90%	1,88% (1)	2%	1%	(1)2% for pensioners
01/01/2009	8,20%	11 ,50%	1,88%	3%	1%	
01/01/2010	8,20%	12 ,50%	2,75% (2)	3%	1%	(2)3% for pensioners
01/01/2011	8,20%	12 ,50%	2,75% (3)	4%	1%	(3)4% for pensioners
01/01/2012	8,20%	12 ,50%	2,75%	4%	1%	
01/01/2013	8,20%	12 ,50%	2,75%	4%	1%	
01/01/2014	8,20%	12 ,50%	2,75%	4%	1%	
01/01/2015	8,20%	12 ,50%	2,75%	4%	1%	

Table B.1: Evolution of expenditure amounts and their growth rate

YEAR	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Expenditure Amounts	799 915	894 465	1004 635	1132 642	1273 007	1428 108	1627 377	1874 768	2153 997	2446 025	2679 490
Growth rate in (%)		11,82	12,32	12,74	12,39	12,18	13,95	15,20	14,89	13,56	9,54

Chart B.1: Evolution of Expense Growth Rates

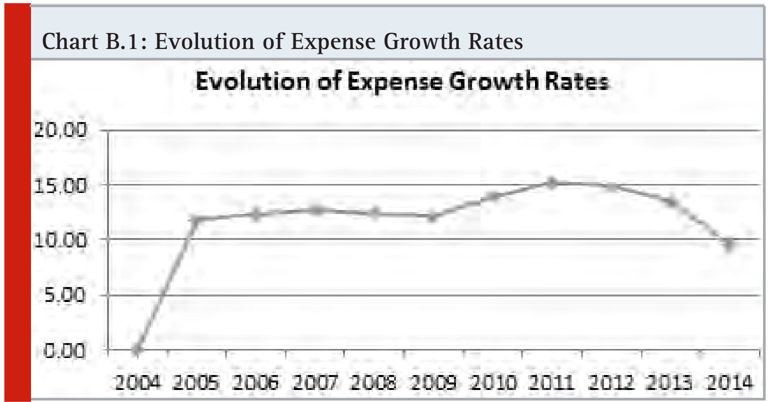
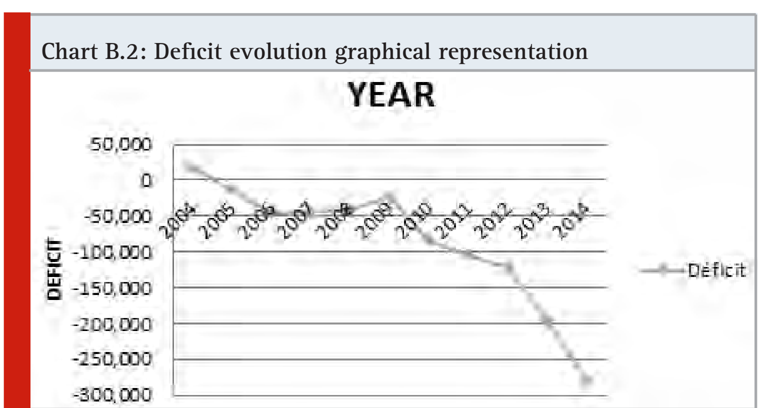


Table B.2: Evolution of receipts and expenditure amounts

YEAR	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Receipts Amount	817 780	881 571	959 416	1089 037	1231 439	1404 820	1542 992	1770 134	2033 110	2250 216	2399 583
Expenses Amount	799 915	894 465	1004 635	1132 642	1273 007	1428 108	1627 377	1874 768	2153 997	2446 025	2679 490
Deficit (MD)	17865	-12894	-45219	-43605	-41568	-23288	-84385	- 104634	- 120887	- 195809	- 279907

Chart B.2: Deficit evolution graphical representation



otherwise they have not moved.

Deficit analysis by expenditure: The evolution of the deficit in terms of pensions: In Table B.1, expenditure amounts have increased annually. It went from 799915 MD in 2004 to 2679490 MD in 2014. Graph B.1 shows that the expense growth rate fluctuates from one year to the next. It is 11.82% in 2005, 15.20% in 2011 and 9.54% in 2014.

The real evolution of the deficit: Table B.2 includes fluctuations in the fund's annual receipts and expenditure: In Table B.2 and Graph B.2, the deficit has increased annually from 12,894MD in 2005 to 279,907MD in 2014. The evolution of the deficit by the demographic factor: The demographic component has a very important role in analyzing expenditure effect on the fund's deficit. In fact, the demographic component represents the mortality table; also known as the life table. This latter is defined as "a construct that makes it possible to follow the fate of a population in detail". This tool is mainly used in demography and actuarial science to study the number of deaths, probabilities of death or survival and life expectancy by age and gender. There are two types of mortality tables: the current mortality table and the generation mortality table where the question arises: what is the difference between the two tables? The current mortality table is constructed on a fictitious generation of 100,000 individuals, then on monitoring the evolution of such a population under the effect of a single elimination factor, namely the mortality factor. We can conclude that in the case of CNRPS, since it is still in deficit (the difference between income and expenditure is negative), it manages this deficit to some extent, either through the monthly reserve of 40 to 50 billion Tunisian dinars that it has at its disposal in the event of late payment by public institutions, or through a delay in payment to the CNAM, or through a loan from the Ministry of Finance. Hence, the present question: Is it possible to mend this deficit? Proposed solutions for the reform of the general pension system: The whole working career is the basis for the pension and the benefit drawn at a given age(Karl Gustaf Scherman : Honorary President of the International Social Security Association; former Director of the National Social Insurance Board in Sweden said it about the Sweden system reform).

In order to control the balance of the general pension system in the long term, a number of solutions should be envisioned to adjust any possible imbalances. This can be done by acting on the factors reducing the revenues of this system, such as, for example, the retirement age limit, the contribution rates, as well as acting on the factors increasing expenditure, like revising the pension calculation parameters. In this regard, we will present solutions that consider

Table C.1.1: The evolution of receipts amounts (2004 - 2010) and growth rate in% after a 1% increase in employer and employee contribution rates

YEAR	Receipts Amount (MD)	Evolution of the growth rate in (%)
2004	817 780	
2005	881 571	7,80
2010	1 542 992	9,84
2014	2 399 583	6,64
2015	2 792 210	16,36
2020	4 705 037	11,00
2025	7 928 261	11,00
2030	13 359 581	11,00
2035	22 511 670	11,00
2040	37 933 474	11,00
2045	63 920 109	11,00
2050	107 709 101	11,00

Table C.1.1: The evolution of receipts amounts (2004 - 2010) and growth rate in% after a 1% increase in employer and employee contribution rates

YEAR	Receipts Amount (MD)	Evolution of the growth rate in (%)
2004	817 780	
2005	881 571	7,80
2010	1 542 992	9,84
2014	2399583	6,64
2015	2 920 884	21,72
2020	4 921 859	11,00
2025	8 293 619	11,00
2030	13 975 230	11,00
2035	23 549 074	11,00
2040	39 681 560	11,00
2045	66 865 736	11,00
2050	112 672 654	11,00

Table C.2.1: The evolution of the impact of the demographic ratio in%

YEAR	Impact on the demographic report
2011	1,00
2012	1,01
2013	1,01
2014	0,89
2015	0,96
2020	0,96
2025	0,96
2030	0,96
2035	0,96
2040	0,95
2045	0,95
2050	0,95

the revenues and expenditure, with 2009 as the base year for any forecast.

Revenue-based solutions: Solution 1: a 1% increase in the employer and employee contribution rates (i.e. instead of 20.70% we have 21.70%): After projections made, we notice that after increasing the contribution rate by 1%, a revenue of 2,792,210 MD will be achieved in 2015 compared to 2,399,583 MD in 2014. Thus, we earned 392,727 MD in one year. Solution 2: a 2% increase in the employer and employee contribution rates, i.e. instead of 20.70%, we have 22.70%: Similarly after the projections made, we notice that after increasing the contribution rate by 2%, we achieve a revenue of 2,920,884 MD in 2015 against 2,399,583 MD in 2014. As a result, we gain 521,301 MD in one year. Expenditure-based solutions: Solution 1: The legal retirement age is set to 62 instead of 60:Similar to our analysis of the revenue-based solutions, extending the

Table C.2.2: The evolution of expenditure amounts and their average, annual equalization amounts and their average, retirement amounts and their average, the effect of equalization (in MD) and their annual and average growth rates in%

YEAR	2008	2009	2010	2011	2012	2013	2014
Expenses Amount in MD	1 273 007	1 428 108	1 627 377	1 874 768	2 153 997	2 446 025	2 679 490
Average expenses in MD				1 926 110			
Growth rate of expenditure amounts in (%)		12,18	13,95	15,20	14,89	13,56	9,54
Average growth rate of of expenditure amount in (%)				13,22			
Equalization effect in MD	44 573	119 823	112 377	145 556	164 334	117 170	44 432
Average equalization effect in MD	1			06 895			
Equalization effect growth rate in%		6,50	15,80	14,14	15,06	17,05	13,15
Average Equalization Effect Growth Rate in%				13,62			
Evolution of the equalization effect in (%)				5,55			
Evolution pensions without equalization effect in MD	1 228 434	1 308 285	1 515 000	1 729 212	1 989 663	2 328 855	2 635 058

retirement age, we notice that until the year 2050, there are 0.96 working people, i.e. only one active person to pay a pension for a single pensioner as projected in Table C.2.1. below: Looking into the table of changes in expenditure amounts and their average, the annual equalization amounts and their average, the retirement amounts and their average and the effect of equalization amounts and their annual and average growth rates (%), we notice that the growth rate of the retirement amounts effect in (%) has increased annually. It rose from 6.50% in 2009 to 13.15% in 2014. To examine the evolution of expenditure during 2015 and 2016, a rate of 5.55% was set for the evolution of the equalization effect, which is an average rate, and then an average growth rate of the retirement amounts effect was set at 13.62% to make projections until 2050.Accordingly, the table below shows that expenditure (in MD) evolved from 2015 to 2050. We notice that for the two years 2015 and 2016 and by increasing the rate of the equalization effect, expenditure amount increased by 147,372 MD in 2015, by 302,849 MD in the following year compared to 2014 and 155,477 MD in 2015 compared to 2016. Therefore, spending has increased annually. Indeed, expense amounts went from 2 679 490 MD in 2014 to 229 097 182 MD in 2050.

Table C.2.3. Expenses evolution (in MD)

YEAR	Solution : Retirement limit age is 62 years Expenses Amount in MD
2014	2 679 490
2015	2 826 862
2016	2 982 339
2020	4 970 227
2025	9 411 311
2030	17 820 671
2035	33 744 110
2040	63 895 739
2045	120 988 982
2050	229 097 182

Tableau C.2.4 : Evolution of pensioners expenses (with a replacement rate of 80%) and an average growth rate set at 13,62%.

YEAR	Solution : Return rate 80% instead of 90% Expenses Amount in MD
2014	2 673 780
2015	3 037 949
2016	3 451 717
2020	5 752 470
2025	10 892 518
2030	20 625 393
2035	39 054 956
2040	73 952 026
2045	140 030 940
2050	265 153 844

Like in the French pension system and 2003 reform (Francois Jeger and Michele Lelievre) for getting more flexibility they with introduce the deductions and bonuses according to duration of contribution with actuarial neutrality. Anyone older than 60 years can retire, with a deduction of 5% per year for fewer than 40 years of contributions and with a bonus of 3% per year for greater than 40 years of contributions.

Solution 2: Revision of pension calculation: The main aim of revising pension calculation is to re-amend or re-improve it to reduce pension expenditure. Referring to our projections and the table below describing the evolution of expenditure for pensioners (with a replacement rate of 80%) with an average growth rate set at 13.62%, we notice that from 2015 onwards, expenditure amount is increasing.

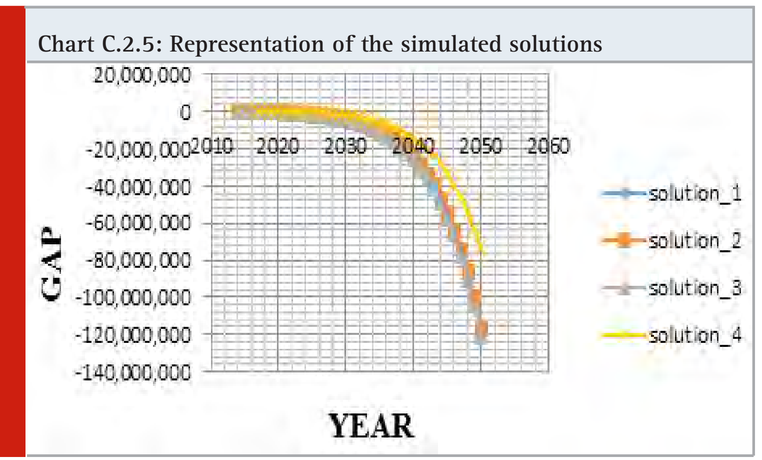
Combined solutions: Below is a summary table that presents the projections of the combined solutions:

- Solution 1: H 3 : 62 years old + an increase in contribution rate 1% (Receipts) + H 1 : The retirement age limit is 62 years old (Expense) In our summary table, for the two years (2016 and 2017), CNRPS will achieve a surplus of 177,890 MD, which will allow it to cover two more years for an amount of 155,750 MD.
- Solution 2: H 4 : 62 years old + an increase in contribution rate 2% (Receipts) + H 1 :The retirement age limit is 62 years old (Expense) In our summary table, for the five years (from 2015 to 2019), CNRPS will achieve a surplus of 793 856 MD, which will allow it to cover another three additional years for an amount of 561 413 MD.

Table C.2.5: Summary table of proposed solutions

YEAR	Solution 1: H 3: Age 62 + raise rate cot 1% (Recipe) + H 1: Retirement limit age is 62 (Expense)	Solution 2: H 4: Age 62 + raise rate cot 2% (Recipe) + H 1: Retirement limit age is 62 (Expense)	Solution 3: H 3: Age 62 + raise rate cot 1% (Recipe) + H 2 : Pension calculation revision + limit age 62 years (Expense)	Solution 4: H 4: Age 62 + raise rate cot 2% (Recipe) + H 2: Pension calculation revision +limit age 62 years (Expense)
2014	-279 907	-279 907	-274 197	-274 197
2015	-30 755	98 098	-24 731	104 122
2016	121 340	264 367	127 695	270 722
2017	56 550	215 310	63 771	222 531
2018	-26 009	150 214	-17 805	158 418
2019	-129 741	65 867	-120 420	75 188
2020	-258 623	-41 499	-248 031	-30 907
2025	-1 471 985	-1 106 117	-1 451 929	-1 086 061
2030	-4 442 445	-3 825 937	-4 404 469	-3 787 961
2035	-11 201 021	-10 162 169	-11 129 112	-10 090 260
2040	-25 909 323	-24 158 797	-25 773 161	-24 022 635
2045	-56 979 662	-54 029 924	-56 721 834	-53 772 096
2050	-121 237 756	-116 267 275	-120 749 550	-115 779 069

Chart C.2.5: Representation of the simulated solutions



- Solution 3: H 3 : 62 years old + an increase in contribution rate 1% (Receipts) + H 2 : Revision of pension calculation + age limit 62 years old (Expense). In our summary table, we forecast that for the two years (2016 and 2017), CNRPS will achieve a surplus of 191 466 MD and which will allow it to cover another 2 years for an amount of 138 225 MD.
- Solution 4: H 4 : 62 years old + an increase in contribution rate 2% (Receipts) + H 2 : Revision of pension calculation + age limit 62 years old (Expense). In our summary table C.2.5, we observe that for the five years (from 2015 to 2019), CNRPS will achieve a surplus of 830,9821MD and which will allow it to cover another 3 years for an amount of 525,113 MD. Then, it seems that solution four is the best solution because its obtained projected figures are much higher than those of the other proposed solutions (solution four's figures(830 9821 MD) are greater than those of solution one (= 177 890 MD), those of solution two (793 856 MD) and those of solution three (= 191 466 MD)). The graph also shows that solution four (yellow line) is the best as a reform solution for the general pension system. It allows CNRPS to cover

more years in terms of deficit. We notice that from 2015 to 2019, the curve has shrunk.

CONCLUSION

As a conclusion, in this paper and thorough analysis, we presented the current situation of the CNRPS and simulated some relevant proposals to mend for the deficit of the general pension system, which has been a serious problem for years and years. This study proposes some solutions to satisfy the expectation and intention of members that impact positively their loyalty. So the CNRPS should have a first class and robust retirement income system that delivers good benefits, is sustainable and has a high level of integrity. (Denmark country system have a grade A with index value >80).

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Synergetic Role of Endophytic Bacteria in Promoting Plant Growth and Exhibiting Antimicrobial Activities

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ABSTRACT

Siderophores Endophytic bacteria are plant associated bacteria living inside the plant tissues symbiotically with damaging the plant tissues. The endophytic bacteria play very important role in plant growth and development by adding variety of metabolites such as phytohormones, antimicrobial compound and other nutrients. In the present study, endophytic bacterium was isolated from Calotropis plant and was identified as *Bacillus sp*. IU103 through 16-sDNA sequencing. The IU103 was analyzed for indole-3-acetic acid through spectrophotometer. Application of IU103 showed that inoculation significantly improved plant growth attributes e.g., root and shoot length, biomass and chlorophyll contents. In addition, IU103 showed antibacterial and antifungal activities. All these characteristics revealed that IU103 has shown some plant growth promoting characteristic and can be used as plant growth promoting bacteria in agriculture.

KEY WORDS: ENDOPHYTIC BACTERIA, INDOLE-3-ACETIC ACID, ANTIBACTERIAL ACTIVITY, ANTIFUNGAL ACTIVITY.

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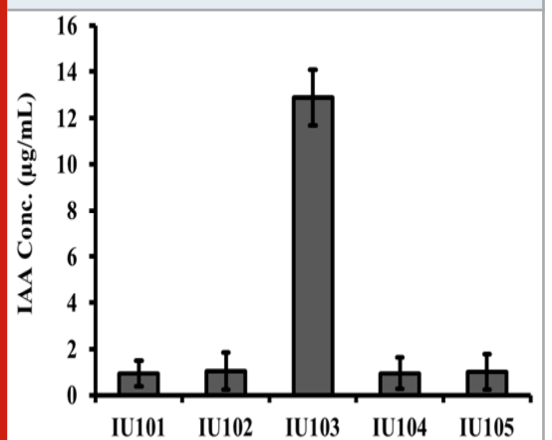
INTRODUCTION

Bacteria associated with plants are called endophytes and they are very helpful to the plants in the term of plant growth promotion and survival under stress to the host plants. Bacterial plant endophytes are existing in a broad species and various of plant organs (Jeger and Spence, 2001; Bharti et al., 2016), without any phenotype changes of plant or decrease of crop yield (Sturz et al., 2000). Numerous studies have described that endophytic bacteria can apply for agricultural purposes with following activities; plant growth promoting (Saharan, 2011), nutrients mobilizing such as nitrogen and phosphorus (Sharma et al., 2013), providing plant hormone such as auxin and gibberellin (Hardoim et al., 2008) and protect the diseases caused by soil-borne pathogens (Berg and Hallmann, 2006).

In addition, endophytes produce numerous distinctive and useful secondary metabolites. Phytohormones are plant growth regulators that conduct signals to control cell division and therefore contribute growth promotion and development of plant (El-Tarabily and Sivasithamparam, 2006). Moreover, they also modulate plant responses to environmental changes, allowing the plant to tolerate environmental stresses (Gouda et al., 2018). Present research have established that certain endophytic microbes, including bacteria, enhance growth of the plants by pouring phytohormones e.g., cytokinins, indole-3-acetic acid (IAA) and gibberellins (Gouda et al., 2018). Bacteria in the genera *Microbacterium, Agromyces, Bacillus, Paenibacillus, and Methylophaga* are reported to produce IAA (Bharti et al., 2016). IAA produced by bacteria, present in the rhizosphere enhances root growth and nutrient availability because of the increased area of fertile soil that is occupied, resulting augmented biomass of the plants resistance against diseases (Ji et al., 2019). Endophytic microbes including bacteria are exploited extensively for the production of novel biologically active compounds such as insecticidal, cytotoxic, and antimicrobial compounds (Kim et al., 2012). The antimicrobial activity of the endophytic bacteria are very important and therefore, these characteristics of the endophytic bacteria have very significant role in medicine and agriculture.

Figure 1. Indole-3-acetic acid (IAA) produced by IU01, IU02, IU03, IU04, and IU05 estimated by spectrophotometer at OD 535 nm.



The secondary metabolites, secreted by bacteria have important application in agriculture (Gouda et al., 2018). In the present study, we have isolated five endophytic bacteria from Calotropis plant. The isolates were screened for idole-3-acetic acid (IAA) production. Further one strain producing high concentration of IAA was selected for further study. Antimicrobial activities of the selected isolate was tested against fungi and bacteria such as *Salmonella enteritidis, Bacillus cereus, Staphylococcus aureus, Escherichia coli*. The overall the endophytic isolate was characterized for IAA production, plant growth promotion, and antimicrobial activity. However, endophytic bacteria should be explored further for their potential benefits in agriculture (Muthukrishnan et al., 2015). Further standardized testing and formulation is needed to discover new endophytes.

MATERIAL AND METHODS

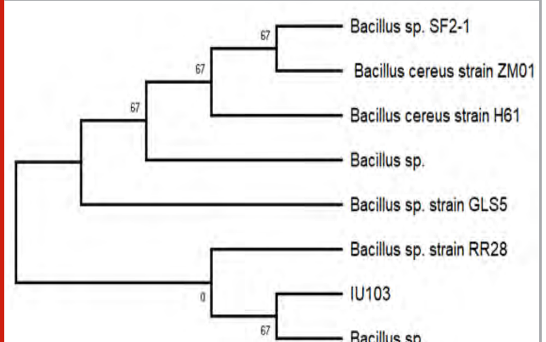
Isolation of bacterial endophytes: *Calotropis* plant were collected and cut off into small pieces and all the pieces were washed for 10 minutes in running tap water to remove the debris, soil particles. After washing, with tape water, the samples were washed with deionized water and out on sterilized tissue paper. Furthermore, plant

samples were dipped in 75% ethanol for 2 minutes followed by dipping in 1% of sodium hypochlorite (NaClO) for 1 minutes again. The samples were finally rinsed with deionized water for 5 times. Eventually the samples were incised through aseptic surgical blade and were placed on LB agar plates. The plants were incubated at 37°C to get the colonies bacteria.

Growth conditions of bacterial culture: In order to grow the endophytic bacteria, LB broth was made containing 10 g yeast extract (difco) 10 g/L; NaCl 10 g/L, peptone, 5 g/L and difco agar 15 g/L. The pH 7was adjusted using 0.01M HCl and/or 0.01M NaOH and the media was autoclaved. The autovalve medium was poured into plants and let the media solidified. The plants were incubated at 37°C for overnight to check the contamination. Eventually the samples were incised through aseptic surgical blade and were placed on LB agar plates. The plates were incubated at 37C for 48 h and visible bacterial colonies were detected.

Calorimetric analysis of bacterial IAA: A test tube of 5 mL LB broth, supplemented with 0.2 g/L of L-tryptophan (Trp) was inoculated with isolates. The culture media was incubated at 30°C on shaker 200 rpm for 72 h. after 72 h, the centrifugation for 12 min was carried out at 10,000 ×g for and supernatant were obtained. One milliliter of culture supernatant and 2 mL Salkowski's reagent (Ullah et al., 2013) were mixed and were incubated at room temperature for 30 min in dark. Addition of Salkowski's reagent and incubation of supernatant generated red color which was measured by spectrophotometer at 535 nm. The concentration of indole-3-acetic acid was estimated in comparison with standard curve made up of IAA (Sigma-Aldrich).

Figure 2. Phylogenetic assessment of 16S rDNA from IU103 with other bacterial strains obtained by BLASTN. The sequences of the bacteria were aligned for construction of phylogenetic tree using MEGA7.



Antibacterial activity fungal Endophytes: The isolate was subjected for antibacterial activity using disc fission dual culture technique. In the first step, pathogenic bacterial suspension (48 h old) was poured on LB agar plate. The isolate, from the culture plate was picked and stabbed on LB plate having pathogenic bacteria. The plate was incubated for 48 h at 37°C. Calculation of the antibacterial activity done by measuring the inhibition zone, produced by endophytic bacteria against pathogenic bacteria.

Antifungal activity endophytes: The isolate was subjected for antifungal activity using disc fission dual culture technique. In the first step, pathogenic fungi was grown on potato dextrose agar plates. After 48 h of incubation at 32, the isolate, from the culture plate was picked and stabbed on BDA plate having pathogenic fungi. The plate was incubated for 72 h at 32°C. Calculation of the antibacterial activity done by measuring the inhibition zone, produced by endophytic bacteria against pathogenic fungi.

Extraction of DNA for PCR analysis: Bacterial cultured grown at 37C for 24 h was used extraction of genomic DNA. The culture was then centrifuged at 10,000 rpm and pellet was used added within 1 mL of extraction buffer containing; 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 2% cetrimide, 1% SDS, 0.7 M NaCl, and 50 μL β-mercaptoethanol. The mixture was incubated at 65°C for 1 h and then mixed with equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated using isopropanol and pellet was washed with 70% ethanol pellet was suspended with 100 μL TE buffer. The polymerase chain reaction (PCR) was used to amplify the DNA through universal primers targeting the 16S rDNA, 27F (5'-AGA GTT TGA TC(AC) TGG CTC AG-3') and 1492R (5'-CGG (CT)TA CCT TGT TAC GAC TT-3'). The PCR was run with conditions: 98°C; 5 min, 97°C; 1 min, 54°C; 30 s, 72°; 1 min,

and a final 72°C for 5 min and total 30 cycles were run.

Plant growth promoting assessment of bacteria: Effects of bacterial endophyte on Brassica plants was determined to estimate the value of bacterial endophytes plant growth promotion. Brassica seeds were dipped in 75% ethanol for 2 minutes followed by dipping in 1% of sodium hypochlorite (NaClO) for 1 minutes again. The seeds were finally rinsed with deionized water for 5 times and the placed on wet autoclaved filter paper on the petri-plates. The seed were kept in moist by adding distilled water after interval of 12 h. the germinated seeds planted in pots filled with autoclaved soil. The pots were placed at 28°C in controlled environment of temperature and humidity (60% relative humidity) in growth chamber. The alternate day night duration was 8 h/16 h respectively with 1600 lx of light for plant growth. Bacterial isolate was cultured in LB broth and was incubated 72 h on shaking incubator at 37°C (160 rpm). The culture was centrifuged at 10,000 rpm at 4°C for 5 min and cell pellets were collected and then suspended in 0.8% saline water (sterile). Plants were inoculated with various concentration of Cd from 0 to 30 mg/mL with concentration difference of 10 mg/mL at the time of seeding.

RESULTS AND DISCUSSION

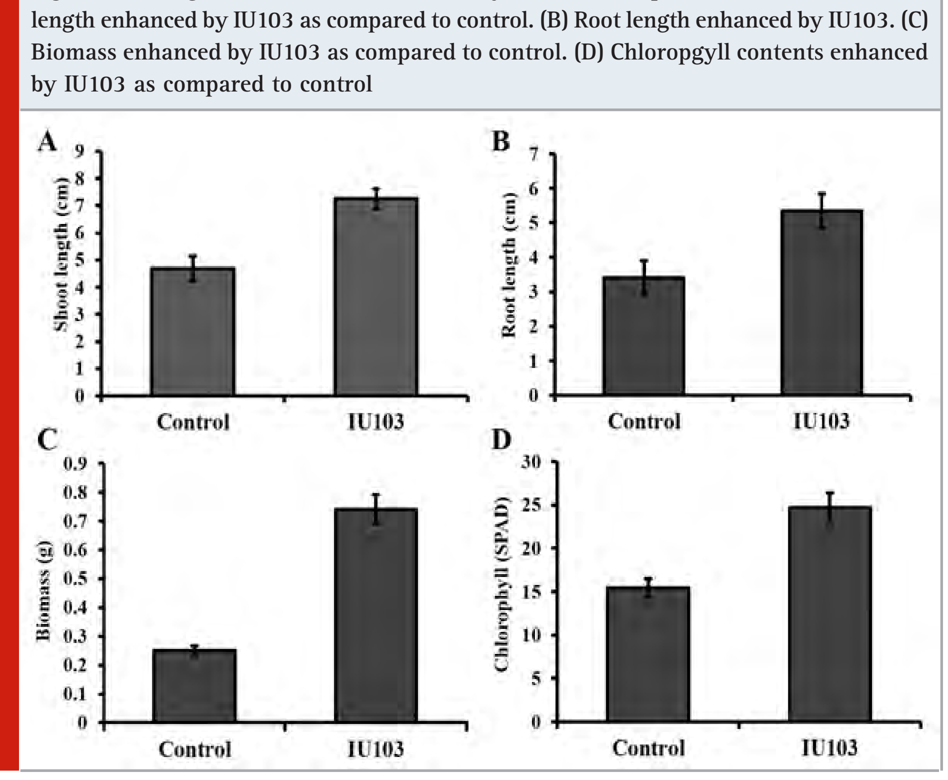
Bacteria isolation

Total five entophytes were purified from the mixed culture. The isolates were primarily purified by picking a single colony from mixed culture and streaking on fresh LB agar plate. Furthermore, the isolates were identified through their morphological characteristics, e.g., color, structure, texture, shape and size of the colonies. The isolates were encoded as IU100, IU102, IU103, IU104 and IU105. All the five isolates were obtained from plant were screen for IAA production through Salkowski's test.

Characterization of IAA production potential

Production of IAA was assessed in all the five bacterial isolates through Salkowski's test. Results

Figure 3. Plant growth attributes enhanced by IU103 as compared to control. (A) Shoot length enhanced by IU103 as compared to control. (B) Root length enhanced by IU103. (C) Biomass enhanced by IU103 as compared to control. (D) Chloropgyll contents enhanced by IU103 as compared to control



of all the five isolates were uneven and color production (indication) of presence of IAA was week in four isolates. The color development in four e.g., IU100, IU102, IU104 and IU105 was not encouraging even after 30 min of incubation in dark. However, IU103, produced reddish color after 30 min of incubation. The results (Fig 1) showed that isolates IU100, IU102, IU104 and IU105 produced negligible amount of IAA which was non-significant. However, IU103 produced significantly high concentration of IAA in culture media.

Analysis of the present data revealed that endophytic bacteria isolated from *Silybum marianum* had significantly enhanced the root and shoot length in heavy metal stress conditions (Bharti et al., 2016; Timmusk et al., 2017). They concluded that endophytes play a key role in the growth of plants under heavy metal. Similarly it proved vital for the increase in fresh and dry weights of the selected plants. The endophytes including fungi and bacteria have been of great interest as inoculants in agriculture to improve plant growth in many crops (Chauvin et al., 2017; Timmusk et al., 2017). Endophytic bacteria enhance plant growth by production of phytohormones such IAA, which play a central role in cell enlargement, root initiation, and cell division (Khan, 2019). Among the tested endophytic isolates, IU103 was producer of IAA. The bacterial isolates might be involved in plant growth promotion due to the IAA production the enhance plant growth by cell enlargement, root initiation, and cell division (McShan et al., 2015) (Ramesh et al., 2015). It has been speculated that production of IAA by bacteria could enhance the legume-Rhizobium symbiosis and uptake of nutrients thus increasing root length and subsequently enhance plant development (Ullah et al., 2015).

Molecular identification of selected bacterial strains: The purified PCR products of 16S rDNA from the IU103, was sequenced, revealing nucleotide sequence lengths. The sequences was aligned with sequences identified by BLAST search of the NCBI database. The selected sequences showed the highest query coverage and sequence homology with IU103. Results of the BLAST sequence search (Fig. 2) indicated the endophyte isolate IU103 showed maximum similarity with *Bacillus sp*. therefore, it was named as *Bacillus sp*. strain IU103.

Plant bioassay of IU103: Effect of IU103 on plant growth attributes of were determined using Brassica plants. Results (Fig. 3A and 3B) showed that inoculated with the IU103 significantly promoted plant growth attributes such as shoot and lengths root length as compared to control. Chlorophyll contents of the plants were an important indicator of photosynthesis, which is necessary for the survival of the plant. Inoculation of IU103 significantly increased the chlorophyll contents of plants and increased plant biomass as compared to control (Fig. 3C and 3D). In short, overall results revealed that inoculation of IU103 significantly affected plant growth, development and application of IU103 was effective, and consistently improved plant growth attributes, e.g., shoot length, root length, chlorophyll content, and biomass as compared to control. The ability of isolates to inhibit the growth of different pathogens is implication of the secondary metabolites secreted by endophytic bacteria (Ullah et al., 2019). It is also evident from the present study that some endophytes isolated can be developed as potential biocontrol agents (Ramesh et al., 2015; Ullah et al., 2019). Therefore, further studies are necessary to assess the ability of the isolates to confer protection against pathogens. The overall study revealed

Table 1. Antibacterial activity of endophytic bacteria IU103.

Treatment	Antibacterial activity of IU103				
	E. coli	*S. typhi*	*B. subtilis*	*S. aureus*	*P. vulgaris*
IU103	+ ve	+ ve	+ ve	+ ve	+ ve
Positive Control	+ ve	+ ve	+ ve	+ ve	+ ve
Negative control	- ve	- ve	- ve	- ve	- ve

Table 2. Antifungal activity of endophytic bacteria IU103.

Treatment	Antifungal activity of IU103				
	A. niger	*A. avamori*	*T. konningi*	*F. oxysporium*	*P. fumicalsuri*
IU103	+ ve	+ ve	+ ve	+ ve	+ ve
Positive Control	+ ve	+ ve	+ ve	+ ve	+ ve
Negative control	- ve	- ve	- ve	- ve	- ve

that the IU103 was effective endophyte, producing high concentration of phytohormone IAA. The IAA production could be responsible for the plant growth promotion. In addition, IU103 also had antibacterial and antifungal activities which also support the plant growth promotion my killing pathogens.

Antibacterial Activity: Antibacterial activity of IU103 was assessed against Gram-positive and Gram-negative bacteria. IU103 was screened for their antibacterial capability against four common Gram-positive bacteria, such as. Similarly, IU103 was also assessed for its antibacterial activities against Gram-negative bacteria. The results revealed that (Table 1) antibacterial compounds were produced by IU103, which were equally effective against Gram-negative and Gram-positive bacteria. Formation of clear zones around the IU103 colonies in culture plates were indication of antibacterial activities of the IU103. *E. coli* DH5 α was used as negative control and tetracycline was used as positive control. Antimicrobial activities have been detected in numerous endophytic bacteria and fungi, which have very important for the plant growth promoting activities because pathogenic microbes such as bacteria and fungi are killed by and hence the plant are protected from their hazardous effects (Ullah et al., 2013, Xia et al., 2016). Moreover, antimicrobial activities of the endophytic microbe revealed that elimination of pathogenic microbe around the plants results pant growth development (Xia et al. 2016). In the present study, among the five bacterial endophytic strains IU103 was found effective against all the tested pathogens.

Antifungal Activity: The inhibitory effect of IU103 was tested against the fungal phytopathogens. Antifungal activity of IU103 was assessed different fungi. IU103 was screened for their Antifungal capability against different species of fungi (Table 2). The results showed that the antifungal compounds were produced by IU103, which were effective against different species of fungi. Formation of clear zones around the IU103 colonies in culture plates were indication of antifungal activities of the IU103. E. coli DH5α was used as negative control and tetracycline was used as positive control. Results of these study revealed that the IU103 showed excellent anti bacterial and antifungal activities. Previously a number of studies have reported antimicrobial activities of plant endophytic bacteria (McShan et al., 2015). Ullah et al., (2015) reported that endophytic bacteria isolated from Solanum sp. Showed antibacterial activities against Gram-negative bacteria (Ullah et al., 2019).

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Conflict of Interest
The authors declare that there is no conflict of interest to disclose.

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Influence on Diabetic Pregnant Women with a Family History of Type 2 Diabetes

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ABSTRACT

Gestational diabetes mellitus (GDM) is defined as diabetes that only progresses during pregnancy and resolves after delivery. Family history (FH) of type 2 diabetes mellitus T2DM is one of the major risk factors for GDM. The aim of this study was to compare GDM between women with and without FH of T2DM. In this study, 137 GDM;150 non-GDM women were involved. Anthropometric and biochemical data were recorded. Calculation analysis confirmed the negative impact with the FH of T2DM between GDM (58.4%) and non-GDM (56%) women (p=0.97). Negative association was observed when the GDM cases with family history of T2DM was compared with GDM without family history of T2DM (p=1.01). However, age, FBG, and PPBG levels were p<0.05; BMI was non-significant (p=0.22). In conclusion, this study disclosed FH of T2DM did not affect GDM in women in an Indian population.

KEY WORDS: GESTATIONAL DIABETES MELLITUS, FAMILY HISTORY, TYPE 2 DIABETES MELLITUS, OBESITY, INDIAN POPULATION.

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INTRODUCTION

In biochemical terms, Gestational diabetes mellitus (GDM) is well-defined as glucose intolerance identified during pregnancy (Ravnsborg et al., 2019). In traditional terms, GDM is defined as the initial detection of hyperglycemia during pregnancy (Agha-Jaffar et al., 2019, Xie et al., 2019). Later in life, GDM women will increase the risk of obesity, type 2 diabetes mellitus (T2DM) or metabolic disorders (Khan et al., 2014). GDM-risk is eight times advanced in obesity pregnant women (Ravnsborg et al., 2019). Advance maternal age, family histories of GDM and T2DM, and western lifestyle have also been documented as risk factors for GDM (Khan et al., 2014). Till now controversies raise for diagnostic criteria which is further needs for treatment and monitoring of GDM; however, omics studies documented regarding the maternal metabolic profile in GDM and non-GDM women, which can be helpful in predicting the GDM disease (Souza et al., 2019). Obesity, hypertension, and physical activity are modifiable-risk-factors while age, ethnicity, and family history (FH) are non-modifiable risk factors of GDM (Khan et al., 2019, Rodrigues et al., 2019).

During pregnancy, a woman's body undergoes numerous metabolic changes; for example, placental hormones stop proper functioning of insulin, leading to insulin resistance, and this results in accumulation of glucose in the blood (Ghassibe-Sabbagh et al., 2019). Presently, no prevention strategy or treatment is available for GDM. However, some lifestyle interventions, such as diet, exercise, and occasionally, insulin therapy, are available for abnormal glucose values during pregnancy. Currently, long-term oral antidiabetics (glyburide and metformin) are promising candidates for long-term treatment of both mother and children (Plows et al., 2018). GDM is detected by GCT/OGTT. World Health Organization presently recommends that OGTT tests be performed during fasting (92 mg/dL), 1h (180 mg/dL), and 2h (153 mg/dL). GDM is diagnosed when the results of any of the tests cross the normal or prescribed values (Kautzky-Willer et al., 2018). In our previous study, we performed OGTT tests during fasting and 1, 2, and 3 h after meals (Khan et al., 2014). Women with GDM can develop T2DM later in life. Their children can be macrosomic at birth or have glucose intolerance in adulthood (Rahman et al., 2019). Additionally, they are prone to obesity from childhood. The risks of GDM, T2DM, and obesity are inter-connected (Lowe et al., 2019). Women should be more careful about these diseases. Genetic predisposition is a risk factor for metabolic disorders; family history plays an important role in medical history. Lifestyle, non-nutritious diet, and environmental factors are other factors that makes the genetic predisposition as an important factor apart from the family histories (Alharithy et al., 2018). Numerous studies have evaluated GDM in Indian populations, but no studies have evaluated the role of family history in GDM development. Thus, this study aimed to compare women with GDM who did or did not have family history of T2DM in an Indian population.

MATERIALS AND METHODS

Recruitment of pregnant women:Ethical approval was obtained to include GDM and non-GDM women in this study. Enrolled 137 pregnant women were diagnosed with GDM and 150 pregnant women as non-GDM. All pregnant women were initially screened by performing glucose challenge tests (GCTs) and OGTTs. Diabetes during pregnancy was confirmed based on abnormal glucose values either in GCT or OGTT. The details of GCTs and OGTTs and their normal and abnormal values were documented in the previous publication (Khan et al., 2014). The criteria for inclusion and exclusion of women with and without GDM were given in recent publication (Khan et al., 2019). Questionnaire:The questionnaire used in this study collected of anthropometric, biochemical, and clinical details as well as family history of T2DM. Age (years), height (cm), weight (kg), mean gestational age of women with GDM, and BMI were calculated. For determining FBG and post-prandial blood glucose (PPBG) levels and for performing GCT and OGTT (fasting hour and 1, 2, and 3 h after meals), fasting and non-fasting coagulant blood was collected and the serum was separated; this serum was used for biochemical analysis. Clinical data consisted

of information about whether the women with GDM were receiving insulin treatment or dietary interventions.

Statistical analysis: The data of GDM cases and controls was analyzed by sample t-tests using R-software system.

RESULTS AND DISCUSSION

Analysis results of clinical factors of women with and without GDM are shown in Table 1. GDM women had ages ranging from 22 years to 38 years, with the mean age of 26.7±5.1 years. Women without GDM had ages ranging from 17-34 (24.6±3.5) years. Mean age was p=0.0001. Weight (69.3±10.1 vs 66.2±9.2 kg) and BMI (26.8±3.9 vs 24.1±3.5 kg/m2) were high in GDM women (p=0.26-0.22). Mean gestational age (24.4±5.0 years) and medication-related factors, such as diet (40.9%) and insulin levels (59.1%) were measured only in women with GDM. Serum glucose factors, such as FBS (110.6±3.9 mg/dL vs. 99.2±11.3 mg/dL) and PPBG (158.8±47.7 vs 112.0±39.7) levels were significantly different between women with and without GDM (p<0.05). FH of T2DM was more frequent in women with GDM (58.4%) than in women without GDM (56%); however, this difference was not significant (p=0.97). In the GDM group, 80 women had FH of T2DM whereas 57 did not have FH of T2DM or any other diabetes. There was no deceptive differences in either GDM with FH of T2DM (n=80) or GDM without FH of T2DM (n=57; Table 2). In the GDM group, mean age was found be higher in women with FH than in women without FH of T2DM (27.3±4.9 vs 25.9±4.4 years; p=0.39). In the GDM group, 51 (63.7%) and 29 (36.3%) women with FH of T2DM were receiving insulin therapy and dietary interventions, respectively; of the women without FH of T2DM, 30 (52.6%) and 27 (47.4%) GDM were receiving insulin therapy and dietary interventions, respectively. This is the first study to compare the risk of GDM according to family history of T2DM in women in an Indian population. Study results indicate the negative association when compared the risks with age and medications (Rx) with (n=80) and without (n=57) family history of T2DM. History of specific disease(s) in the family or family pedigree can affect the presence of genetic diseases in any individual. For example, genetics or family pedigree can affect diabetes. Among complex diseases, T2DM is mainly influenced by genetic and environmental factors; a similar influence is observed on obesity, which is also known as an important predictor of development of T2DM and cardiovascular diseases (CVDs). FH of T2DM is a

Table 1. Comparison of clinical factors between gestational diabetes mellitus (GDM) cases and controls

Clinical Factor	GDM (n= 137)	Non-GDM (n=150)	P-value
Age (years)	26.7±5.1	24.6±3.5	0.0001
Weight (cm)	69.3±10.1	66.2±9.2	0.26
BMI (Kg/cm^2)	26.8±3.9	24.1±3.5	0.22
Mean gestational age (years)	24.4±5.0	NA*	NA*
Fasting blood glucose (mg/dL)	110.6±3.9	99.2±11.3	<0.0001
Post- prandial blood glucose (mg/dL)	158.8±47.7	112.0±39.7	0.02
RX (Diet/ Insulin)	40.9%/59.1%	NA*	NA*
Family history of type 2 diabetes mellitus	58.4%	56%	1.01

Na= Not analyzed and not applicable

well-known risk factor for GDM, is known to be a medical condition equally appears in T2DM. Both T2DM and GDM have similar pathophysiologies that involve β-cell dysfunction and insulin resistance (Khan et al., 2014). FH has been known to be a risk factor for CVD and T2DM, and specific diseases reflect the consequences of genetic susceptibility, shared environment, and common behaviors. In the field of clinical medicine, FH is recognized as an important but not yet non-modifiable risk factor diseases prevents the influences of suspected diagnosis. The major advantage of FH over genomic tools includes the low cost of determination of genetic and environmental risk factors; however, this utility has been poorly explored in public health (Das et al., 2012). FH of T2DM is a strong metabolic risk factor of T2DM. The major risk factors of T2DM are waist circumference, BMI, physical inactivity, and family history (Diabetologia, 2013). FH of diabetes in first-cousin consanguinity is an initial risk factor for GDM; this further emphasizes the role of genetics in diabetes susceptibility (Kiani et al., 2017).

GDM is associated with maternal obesity, which confers a 4-7-fold risk of T2DM occurrence along with a predisposition toward development of MetS in middle age. FH of GDM in women without diabetes is characterized by increases in FBG and insulin levels, dyslipidemia, and high inflammation in the absence of MetS. Self-histories of GDM women is having the future risks with MetS and 66% of CVD such as stroke, myocardial infarction and coronary artery disease. However, no global studies are available that support the women diagnosed with GDM in the first delivery has increased with MetS and CVD (Gunderson et al., 2014). Moreover, T2DM negates the protected effect in females, but the CVD risk is comparable between men and women with T2DM. MetS and CVD alters the increases T2DM risks which is characterized by adiposity, dyslipidemia, hypertension and elevated fasting glucose levels. Several studies have reported evidence of the MetS in women with a history of GDM (Carr et al., 2006). Limited risk factors were documented in the GDM women. However, the importance of other factors, such as short stature, lower birth weight, ethnicity, smoking status, multiparity, gestational weight gain, physical inactivity, and socioeconomic factors, is still controversial (Dode and Santos, 2009). Carr et al (Carr et al., 2006) concluded that CVD was significantly common in women with history of GDM. Dode et al (Dode and Santos, 2009) performed a meta-analysis study by considering age, FH of T2DM, and obesity as well-identified risk factors in GDM women and concluded that publication bias cannot be ruled out with small sample sizes. Kiani et al (Kiani et al., 2017) performed a systematic review and meta-analysis on women with GDM and confirmed that age, obesity, and family history of T2DM were risk factors for GDM in Iranian women.

In subjects without metabolic disorders, physical activity has been reported to reduce the risk of diseases and unnecessary fat accumulation, weight gain, insulin resistance, and specifically diabetes. The relation between physical activity and GDM during pregnancy has been stated as lower levels of glucose intolerance (Bung et al., 1991). Aune et al (Aune et al., 2016) performed a meta-analysis on GDM women performing physical activity and confirmed

Table 2. Assessment of subjects with gestational diabetes mellitus (GDM) with and without family history of T2DM

Selected clinical factors	GDM with FH(n= 80)	GDM without FH(n=57)	P-Value
Age (years)	27.3±4.9	25.9±4.4	0.39
RX (Insulin/Diet)	51(63.7%)/29(36.3%)	30(52.6%)/27(47.4%)	0.57
Family history of T2DM	80(100%)	57(100%)	0.99
FH=Family history			

the significant inverse association. Previous studies involving American and Australian women with GDM have confirmed that FH of T2DM is a risk factor, along with other features (Santos-Ayarzagoitia et al., 2006). Other global studies have also confirmed that FH of T2DM is one of the risk factors in women with GDM (Herath et al., 2017, Kuti et al., 2011, Lin et al., 2016, Moosazadeh et al., 2017, Ogonowski et al., 2014, Rhee et al., 2010, Van Leeuwen et al., 2010, Wahabi, 2018, Yang et al., 2009). However, Mahalakshmi et al (2014) performed a study in India and concluded that 70% of the enrolled subjects with GDM had FH of T2DM. Current study results are consistent with those of Riaz et al (Riaz et al., 2018) who did not find FH of T2DM to be a risk factor in women with GDM. Schleger et al (Schleger et al., 2018) concluded that FH of T2DM in the mother affected fetal post-prandial brain function. This study concludes that FH of T2DM is not a risk factor in women with GDM in an Indian population, when compared with either women with GDM who had no FH of T2DM or women without GDM. This could be due to the limited sample size in the capital city of diabetes mellitus.

Conflict of Interest: There is no conflict of Interest towards this article.

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Remediation of Animals, Plant and Insect toxic metal, Cadmium through Hyperaccumulator Plant, *Solanum nigrum*

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ABSTRACT

Cadmium (Cd) is highly toxic to animal, plant and agricultural important insects. Hyperaccumulator plant, *Solanum nigrum* L. was exposed to different concentrations of (Cd) such as 10 mg/kg and 50 mg/kg of soil. The results revealed that concentration of Cd reduced plant growth attributes such as shoot length and root length in a dose-dependent manner as compared to control. The reduction of growth attributes was significant ($p < 0.05$) at 50 mg/kg of soil as compared to 10 mg/kg of soil and control. Application of Cd also reduced the chlorophyll contents, fresh and dry biomass significantly ($p < 0.05$) as compared to control. The assessment of Cd accumulation in shoot and roots through inductively coupled plasma mass spectrometry (ICPMS) analysis showed that a higher concentration of Cd was accumulated in roots as compared to shoot. The study concluded that S. nigrum has potential to be used in Cd-contaminated marginal land for phytoremediation.

KEY WORDS: PHYTOREMEDIATION, CADMIUM DETOXIFICATION, SOLANUM NIGRUM, GROWTH ATTRIBUTES.

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INTRODUCTION

The presence of heavy metals in the environment has become a major threat to plants, animals and human life due to their tendency of bioaccumulation and toxicity in living organisms (Bahadir et al., 2007). Larger agricultural soil has been contaminated with heavy metals due to mining activities, industrial discharges and the application of agrochemicals and lime products. Contamination of soil with heavy metals enhances plant uptake leading to accumulation in various parts of the plants (Bahadir et al., 2007; Ullah et al., 2019). From soil to plant, transport of heavy metals depends on total amount of heavy metals, ionic ratios of elements in soil solution and rate of transfer of element from solid to liquid stages and to plant roots (Buendía-González et al., 2010). Severe heavy metal contamination in the soil may cause a variety of problems, including low crop yield and toxicity in plants, animals, and humans. A trace amount of few heavy metals such as Cu and Zn are essential for plant growth and natural development because they are used as co-factors for many enzymes (Ullah et al., 2015).

However, high concentrations of both essential and unnecessary heavy metals in the soil can lead to toxic symptoms and growth inhibition in most of the plants (Dahmani-Muller et al., 2000). At the cellular level, excessive amounts of toxic heavy metal ions stimulate many stress responses and damage various cell components such as cell membranes, proteins and nucleic acid (Fidalgo et al., 2011; Chauvin et al., 2017).

Plant treatment refers to the natural ability of certain plants called hyper-accumulator that accumulate the pollutants and either immobilize them or turn them harmless to the soil, water or air. Contaminants such as minerals, pesticides, solvents, explosives, crude oil, and derivatives have been detoxified by plant treatment known as phytoremediation (Buendía-González et al., 2010). *Solanum nigrum* has been successful in accumulating highly toxic pollutants at pollutant sites (Fidalgo et al., 2011). Phytoremediation technique is a method of treatment that takes advantage of the ability of hyper-accumulator plants to accumulate heavy metals and toxic compounds from the environment and metabolize them in their tissues (Garbisu and Alkorta, 2001).

S. nigrum is a fast-growing plant that produces high biomass even in soil contaminated with heavy metals. Thus, this plant may be effective in plant filtration or plant stability of wastewater contaminated with heavy metals. To date this type of plant has been used in studies of the effects of heavy metals such as cadmium hyper-accumulator (Ullah et al., 2013; Xia et al., 2016). The aim of this study is to know the absorption of heavy metals by plants and their effect on the content hyperaccumulator plants. *S. nigrum* was selected because it accumulates heavy metals in higher concentration and known as Cd hyperaccumulator plant.

MATERIALS AND METHODS

Seed germination and pot experiment: Seeds of

Table 1. Growth attributes of *Solanum nigrum* L. with different concentrations of Cd

Cd Treatment (mg/kg)	Shoot length (cm)	Root Length (cm)	Chlorophyll contents (SPAD)
0	10.26 ± 2.02[a]	12.33 ± 0.54[a]	22.11 ± 2.23[a]
10	9.40 ± 1.38[a]	11.00 ± 1.43[a]	18.21 ± 1.67[b]
50	5.38 ± 0.57[b]	6.25 ± 0.56[b]	12.56 ± 2.11[c]

Mean ± SD values are presented in each column different letters represent the significant difference ($p < 0.05$) as analyzed by Duncan's multiple range test.

S. nigrum L. were collected, surface-sterilized and germinated according to the procedure of Wei et al., (2009). The germinated seeds were grown commercial soil and were put in a growth chamber at 25 ± 2 °C. Two different Cd concentrations: 10 mg/kg and 50 mg/kg of soil were used as treatments and 0 mg/kg was (without Cd) was used as control. The experiment lasted for 4 weeks. Three replications were used and ten plants per replication were used in the experiment.

Measurement of plant growth attributes: After 4 weeks, the experiment was harvested and plant growth attributes of Cd treated and control plant was determined. The total length of the plants (Cd treated and control) was measured as root length and shoot lengths. The chlorophyll contents of the treated and control plants were also determined using chlorophyll meter (SPAD- 502, Minolta, Japan).

Fresh and dry biomass assessment: The plants were cut off and roots and shoot were separated. Fresh weight of roots and shoots of the Cd treated and control plants were measured. The shoots and roots were dried separately in paper bags at 100°C for 10 minutes and dried at a constant weight at 70°C.

Assessment of Cd in plant shoots and roots: The oven-dried plant samples (Cd treated and control) were crushed into powder and the 100 mg of crushed samples were digested in a solution of HNO3-HClO4. The concentration of Cd in the digested samples was determined by double plasma spectral analysis (ICP, Optima 79000DV, PerkinElmer, USA).

Table 2. Concentration of Cd in shoot and roots of Solanum nigrum treated with different concentrations of Cd of mg/kg of soil.

Cd Treatment (mg/kg)	Fresh weight	Dry weight
0	60.54 ± 3.22[a]	22.68 ± 1.45[a]
10	40.33 ± 1.53[b]	12.19 ± 0.66[b]
50	15. 83 ± 1.45[c]	9.25 ± 0.56[c]

Mean ± SD values are presented in each column different letters represent the significant difference ($p < 0.05$) as analyzed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Assessment of plant growth attributes: The results revealed that the application of Cd negatively affected the growth characteristics of *S. nigrum* compared to the control (0 mg/kg of Cd). Plant growth attributes of Cd treated plants were not significant ($p < 0.05$) different at 10 mg/kg as compared to control; however, when the concentration reached from 10 mg/kg to 50 mg/kg 50 mg/kg the root lengths and shoot length were significantly decreased as compared to control (Table 1). The magnitude of the growth reduction compared to the control was evident at higher concentrations of cadmium. The length of root and shoot were significantly affected in a dose-dependent manner compared to the length of the control plants. Previous studies have shown that bacterial endophytes isolated from S. nigrum were not only capable of detoxifying Cd but also promoting growth of *S. nigrum* Khan et al. (2015). Based on previous findings, we designed a study to isolate bacterial endophytes from *S. nigrum* and to determine their effects on plants. *S. nigrum* was selected for the experiment because of its tolerance for heavy metals and comparatively short life cycle (Jabeen et al., 2009).

Measurement of chlorophyll content: The contents of chlorophyll present in the plants exposed to Cd from 10 mg/kg to 50 mg/kg were measured. The results revealed (Table 1) that plants there was a significant reduction in chlorophyll contents in plants exposed to Cd concentrations as compared to the control plants. In addition, a significant difference in contents was measured in plants exposed to 10 mg/kg and 50 mg/kg of Cd as compared to the control. The plant growth inhibition by Cd was presumably due to the damage of chlorophylls contents and other photosynthetic pigments by toxic effects of Cd Ullah et al., (2013) suggested that chlorophyll degradation was a major reason for growth reduction in plants exposed to under Cd stress.

Table 3. Fresh and dry biomass of Solanum nigrum after treatment with different concentrations of Cd (mg/kg of soil).Method

Cd Treatment (mg/kg)	Cd Concentration mg/kg of Plant DW*	
	Shoot	Root
0	ND*	ND
10	120.37 ± 3.54	289.23 ± 8.34
50	251.82 ± 5.47	487.23 ± 14.42

ND* = represents not detected and DW* = represents dry weight. The values are expressed as the mean ± SD and different letters represent significant differences ($p < 0.05$).

Fresh and dry biomass assessment: Fresh and dry biomass of the *S. nigrum* plants exposed to the Cd was greatly affected as compared to controlled plants. The fresh weight of the plant exposed to Cd showed a gradual and significant reduction with gradual increase in Cd concentrations. Similarly, the dry weight of the plants was significantly decreased with increased concentration of Cd in dose dependent manner as compared to control (Table 2). Fidalgo et al., (2011) reported similar results; their report revealed that the Cd concentration greatly reduced the biomass of *S. nigrum*, with increased concentration of Cd. In addition, inhibition of growth in S. nigrum plants was assessed by Wan et al., (2012) and fresh and dry biomass were monitored under increasing concentrations of Cd. The inhibition of plant grow under higher Cd concentration was assumed to be mainly due to the effect of heavy metals on the contents of photosynthetic pigments, which are responsible for photosynthesis (Liu et al., 2009; Kurzbaum et al., 2014).

Cadmium accumulation in shoot and root of the *S. nigrum:* The concentration of Cd in shoots and roots of the plant significantly increased in a dose-dependent manner. The Cd concentrations and accumulations in different parts of the plants grown in different Cd concentrations revealed that Cd accumulation in a Cd-exposed plant was significantly higher than that in the control (Table 3). Moreover, the Cd contents increased from 120.37 to 251.82 mg/kg in the shoots and 289.23 to 487.23 mg/kg in the roots. A higher concentration of Cd was accumulated in the roots of S. nigrum as compared to shoots. Same Cd behavior of Cd accumulation was reported by Wei et al. (2013), their report showed that an increase in Cd in the soil was associated with higher amounts of Cd accumulating in roots of the S. nigrum. The studies conducted by Malandrino et al., (2006), Wan et al., (2012). John et al., (2008) showed that Cd accumulation was higher in the roots of plant as compared to shoots. Similarly Wan et al., (2012) reported that the level Cd accumulated in the roots was many-fold higher as compared to the shoots of the *S. nigrum.*

CONCLUSION

Heavy metal such as Cd is highly toxic to plants and animals including human being and Cd contamination has been a major issue in recent decades. Phytoremediation through Cd hyperaccumulator plant *S. nigrum* is an economic and most important technique used to eliminate the Cd from soil. In the present study, *S. nigrum* plant was used in Cd contaminated soil. The study showed the *S. nigrum* successfully accumulated Cd in roots and shoot and eliminated the Cd from the soil.

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Biorefinery Sequential Extraction of Alginate by Conventional and Hydrothermal Fucoidan from the Brown Alga, *Sargassum cristaefolium*

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ABSTRACT

Brown algae has a fucoidan and alginate bioactive components with different characteristics. Brown algae has a great potential as feedstock for biorefinery alginate and fucoidan extraction which is integrated. Alkaline extraction process parameters in the sequential fucoidan and alginate extraction are integrated affected to the characteristics of alginate and fucoidan from brown alga *Sargassum cristaefolium*. This study aims to understand the effect of the parameters of the alkaline extraction process on multiple responses alginate and fucoidan yields, and determine the optimal alkaline extraction process in the integrated alginate and fucoidan sequential extraction processes according to the concept of industrial biorefinery. Box Bhenken Design from the response surface method was used to understand the effect of process parameters temperature, time and Na_2CO_3 levels on the multiple responses alginate (yield, intrinsic viscosity, molecular weight) and fucoidan yield. The results showed that the alkaline extraction process parameters significantly affected on the multiple responses alginate and fucoidan yield with quadratic pattern. The optimal conditions occur at a temperature of 57.02 °C, time of 123.96 min, and Na_2CO_3 concentration of 2.66%. Under the optimal point, the yield of alginate was 34.51 ± 0.87%, intrinsic viscosity was 409.72 ± 7.59 ml/g, molecular weight of alginate was 194.08 ± 3.65 kDa and fucoidan yield was 1.81 ± 0.06%.

KEY WORDS: *FUCOIDAN, ALGINATE, BIOREFINERY, ALKALINE-TREATMENT, SARGASSUM CRISTAEFOLIUM.*

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INTRODUCTION

Brown algae has a fucoidan and alginate bioactive components with different characteristics (Rioux et al., 2007; Torres et al., 2007; Draget and Taylor, 2011). The function of alginates works as thickener and gelling agent, while alginate gels are thermostable (Rahelivao et al., 2013; Sellimi et al., 2015). Alginate is widely used in non-food and food industries (Poncelet et al., 1999; Gomez et al., 2009), in the field of pharmaceutical, alginate is used as a slow release of drugs (pharmaceuticals) and antitumor compounds (Sousa et al., 2007; Moebus et al., 2012; Wang et al., 2010; Jensen et al., 2012). The fucoidan works as an anti-inflammatory, anti-tumor, anti-cancer, and immunomodulatory compound (Asker et al., 2007; Ye et al., 2008; Kim et al. 2010; Ale et al., 2011; Costa et al., 2011).

Brown algae has great potential as a feedstock process of bio-refinery alginate extraction and integrated fucoidan (Jung et al., 2013; Ruiz et al., 2013), however this potential cannot be optimally utilized to produce alginate and fucoidan to meet domestic needs.The problem of the fucoidan and alginate sequential extraction on bio-refinery process is low fucoidan yield. This is because fukoidan extraction with acid treatment is carried out at low temperatures and when done at high temperatures, the results show degradation of the alginate polymer chains that indicates this method is not effective (Sugiono and Ferdiyansah, 2018). Therefore, an alternative extraction is necessary for more effective methods that can produce high yield fucoidan with good quality, namely the conventional alginate extraction bio-refinery process and algae residues as feedstock for hydrothermal extraction of fucoidan.

Alginates works as polar and in sodium carbonate solution, alginate extraction with sodium carbonate solution can produce alginates with high yield and viscosity . While fucoidan is polar soluble in acidic solutions and water. Hydrothermal fucoidan extraction can produce fucoidan with low molecular weight and high yield (Quitain et al., 2013). Low fucoidan molecular weight has higher bioactive properties than large fucoidan molecular weight . This is the basis for the development of the conventional alginate extraction bio-refinery process and hydrothermal fucoidan extraction. However, the effect of the alkaline treatment conditions on multiple responses of alginate and fucoidan through an integrated process is not yet known. Some researchers have used alkaline treatment for sequential extraction of alginate and fucoidan to characterize (Rioux et al., 2007), but it does not refer to the perspective of industrial bio-refinery. Therefore, it is essentially needed for optimal alkali treatment conditions in the bio-refinery extraction process of alginate and fucoidan from brown algae so that it can produce high yields and good quality. In this study optimization parameters of Na_2CO_3, temperature and time of conventional alginate extraction and integrated fucoidan hydrothermal will be carried out.

MATERIALS AND METHODS

Materials and reagents: Brown algae *Sargassum cristaefolium* was obtained from Poteran Island in Sumenep, Madura, and collected in Maret 2019. Chemicals (distilled water, ethanol 99.8%, Na_2CO_3) for extraction and analysis were analytical grade.

Sample preparation: The Brown algae washed with fresh water until it is clean, then dried under the sun to reach 13% moisture content. Dried brown algae are processed with a coffee grinder and filtered with a 60 mesh filter (Lorbeer et al. 2015). Brown algae were immersed in a 96% Etoh for overnight to remove phenol and protein components, washed and dried at 45 °C for 6 hours (Ale et al. 2012).

Sample preparation: 10 g of brown algae were added with Na_2CO_3 solution with a concentration of 1-5%, solvent ratio 1:20 (w/v). Alginate is extracted conventionally with a water temperature of 30-90 °C for 60-180 min (Lorbeer et al., 2015). Then cooled and filtered with a filter press cloth so that the residue A and filtrate are obtained. The alginate filtrate was added with a 96% ethanol ratio of 1: 2 (v / v) left for 2 hours and filtered. Alginate is washed twice with 70% and 96% of ethanol then filtered and pressed, the alginate is dried in an oven at 45 °C for 24 hours and milled on 60 mesh.

Fucoidan extraction: Residue A of alginate extraction was dissolved in 1:60 (w/v) ratio distilled water, and hydrothermally extracted using ECOPAN VITA + Smart Pressure Cooker (90 KPa) for 3 hours. Then the residue and filtrate are separated. Fucoidan filtrate added with ethanol 96% ratio of 1: 2 (v/v) was left overnight, fucoidan were separated by centrifugation speed of 7000 rpm for 10 minutes. The fucoidan are collected and dried with a vacuum dryer at 45 °C for 16 hours (Ale et al., 2012).

Experimental design and statistical analysis: The experimental design used in this study is the Box Behnken Design from the response surface method. The parameters and levels studied were temperature (30, 60, 90 °C), duration (60, 120, 180 min) and Na_2CO_3 levels (1, 3, 5%) coded +1, 0, and -1 (Table 1). Actual variables and codes with 3 central point replications are presented in Table 2, the total number of experiments was 15 treatments (Montgomery, 2005). The central point chosen in this study is the result of previous studies. The yield of alginate extract (%), intrinsic viscosity (ml/g), molecular weight (kDa) and fucoidan yield (%) of the BBD design were analyzed response surface regression and the accuracy of the polynomial model (eq. 1).

$$y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} x_i x_j \quad (1)$$

Yield: Yield was calculated based of ratio between alginate or fucoidan weight over to initial wight of brown algae, and then multiplied 100 % (Lorbeer et al., 2015).

Intrinsic viscosity: Intrinsic viscosity of alginate was determined by using capillary viscometer Ubbelohde (Canon, USA), capillary diameter of 0.56 and employer at temperature 25 °C. 30 mg alginate was diluted in 10 ml aquabides, stirred for 5 hour at room temperature (25 °C), and then made serial concentration of 0.05-0.3 g/dL. Flow time of solution (t) relative to flow time of solvent (t0). The intrinsic viscosity was determined by extrapolating from equation ηsp/c (eq. 5) until zero concentration (Chee et al., 2011).

Relative viscosity, $$\eta = \frac{t}{t_0} \quad (2)$$

Specific viscosity, $$\eta_{sp} = \eta - 1 \quad (3)$$

Reduction viscosity, $$\frac{\eta_{sp}}{c} = \frac{\eta - 1}{c} \quad (4)$$

Intrinsic viscosity, $$[\eta] = \frac{\eta_{sp}}{c} \quad (5)$$

Molecular weight: Alginate molecular weight was calculated based relationship between averaged intrinsic viscosity and molecular weight by using Mark-Houwink equation (eq. 6), where k=0.023 dL/g and a=0.984 (Clementi et al., 1998).

$$[\eta] = kM_w^a \quad (6)$$

Where [η] is intrinsic viscosity in dL/g, Mw = molecular weight in kDa

RESULTS AND DISCUSSION

Alginate yield: The results showed that the extraction process parameters (temperature, time, concentration of Na_2CO_3) on the yield of alginate was obtained yield of alginate within a range between 27.1-40.6% (Table 2). The higher the temperature, extraction time and concentration of Na_2CO_3, the yield of alginate tends to increase with a higher temperature, extraction time, and concentration of Na_2CO_3. The cell walls of brown algae become softer and expand with increasing temperature, time and concentration of Na_2CO_3 so that alginate extractability increases. Fertah et al. (2014) states that the higher the extraction process parameters (temperature and time), the higher the yield obtained until it reaches the optimal point after it decreases. Brown algal cell walls are increasingly porous as a result of higher temperatures and extraction times (Hernadez-Carmona et al., 1999; Torres et al., 2007).

The results of the study (Figure 1) showed that the extraction process parameters had a significant effect ($P < 0.05$) on alginate yield. Alginate yield is higher with the increasing temperature, time and concentration of Na_2CO_3 with quadratic pattern. The highest alginate yield of 40.6% occurred at 90 °C, 120 min and 5% Na_2CO_3 of concentration, while the lowest alginate yield was 27.1% at 30 °C, 180 min and 1% Na_2CO_3 of concentration. The results of this study are aligned with the

Figure 1. Response surface plot of the effect of parameters alkaline extraction process on the alginate yield

mentioned literature (Rahelivao et al., 2013; Lorbeer et al., 2015; Sugiono et al., 2019b).

Intrinsic viscosity: The parameters of the extraction process temperature, time and concentration of different Na_2CO_3 obtained an intrinsic viscosity of alginate that increased at 60 °C, 120 min and concentration of 3% then decreased at 90 °C, 180 min, and 5% of Na_2CO_3 concentration. The highest intrinsic viscosity is obtained for 412.06 ml/g at a temperature of 60 °C, 120 minutes and a concentration of 3%. The lowest intrinsic viscosity at 90 °C, 180 minutes, and 5% of concentration is 65.92 ml/g. The results of this study are in accordance with that reported by Rahelivao et al. (2013), Fenorodosa et al. (2010) and Torres et al. (2009). The parameters of 1-5% Na_2CO_3 concentration, temperature of 30-90 oC and extraction time of 60-180 min had a significant effect (P <0.05) with a quadratic pattern on the intrinsic viscosity of *Sargassum cristaefolium* alginate (Figure 2). Intrinsic viscosity of alginate increases with the higher extraction parameters and then decreases after reaching optimal points.

This matter can be explained by several phenomena: first, increasing the process parameters causes the algae cell wall to expand and soft so that the extraction of alginate of large molecular weight increases. Second, the low intrinsic viscosity at 1% Na_2CO_3 concentration because of the long chain polymer alginate is not extracted, so that the viscosity is low. Third, the decrease in intrinsic viscosity of alginate at 5% Na_2CO_3 concentration is produced as a result of the degradation of the alginate polymer chain (Smidsrod et al., 1969; Haug et a1., 1967). The degradation of the main chain of alginate polymers increases rapidly at 90 oC and 180 minutes (Hernandez-Carmona et al., 1999; Lorbeer et al., 2015; Silva et al., 2015; Sugiono et al., 2019a).

Molecular weight: Biorefinery process parameters extraction on temperature, time and concentration

Table 1. Coded and actual of independent variables

Independent variables	Symbols	Variables	
		Coded	Actual
Temperature (°C)	x1	-1	30
		0	60
		+1	90
Time (min)	x2	-1	60
		0	120
		+1	180
Na_2CO_3 (%)	x3	-1	1
		0	3
		+1	5

Table 2. Box-Behnken Design from RSM and responses

No	Actual variables			Responses			
	Temperature (°C)	Time (min)	Na_2CO_3	Fucoidan yield (%)	Alginate yield (%)	Intrinsic viscosity (ml/g)	Molecular weight (kDa)
2	60	180	1	1.92	30.12	140.15	65.14
3	90	120	1	0.75	28.90	135.73	63.05
4	30	180	3	1.72	28.21	260.21	122.18
6	90	120	5	0.20	40.60	65.92	29.83
7	30	120	1	1.50	27.10	149.61	69.61
8	60	60	5	0.22	27.32	113.45	52.55
9	90	60	3	0.27	29.80	198.61	92.84
10	30	60	3	0.25	27.48	267.92	125.86
12	30	120	5	0.26	29.13	112.55	52.130
13	60	180	5	1.23	36.74	97.32	44.97
14	90	180	3	1.78	35.98	195.40	91.32
15	60	60	1	1.54	29.85	287.42	135.17
1	60	120	3	1.81	35.80	407.21	192.59
5	60	120	3	1.91	34.54	412.06	194.93
11	60	120	3	1.72	33.21	392.56	185.55
Pred.	57.02	123.99	2.66	1.92±0.17a	33.12±0.82b	404.73±18.55c	191.38±8.81d
Valid	57.02	123.99	2.66	1.81±0.06a	34.51±0.87b	409.72±7.59c	194.08±3.65d

Table 3. Polynomial models, significance codes and fitting models

Coefficient	Fucoidan yield (%)	Alginate yield (%)	Intrinsic viscosity (ml/g)	Molecular weight (kDa)
Intercept β_0	+1.81	+31.65	+403.94	+191.02
Linear value β_1	-0.091ns	+2.92*	-24.33ns	-11.59ns
β_2	+0.55**	+2.07ns	-21.79ns	-10.35ns
β_3	-0.48**	2.23 ns	-40.46*	-19.19*
Quadratic β_{11}	-0.68**	-1.86 ns	-108.52**	-51.89**
β_{22}	0.13ns	-2.29 ns	-64.89*	-31.09*
β_{33}	-0.46*	-1.22 ns	-179.47**	-85.48**
Cross product β_{12}	+1.00ns	+1.36ns	+1.13ns	+0.54ns
β_{13}	0.17ns	+2.42*	-8.19 ns	-3.94 ns
β_{23}	0.16ns	+2.29 ns	+32.78 ns	+15.61ns
Fitting model				
P value	0.0145*	0.0205*	0.0578*	0.0022**
Lack of Fit	0.0611ns	0.2778ns	0.7914ns	0.059ns
R2	0.9393	0.9296	0.9723	0.9725

Equation of the type $Y= \beta_0+ \beta_{x1}+ \beta_{x2}+ \beta_{x3}+ \beta_{x1x2}+ \beta_{x1x3}+ \beta_{x2x3}+ \beta_{x1x1}+ \beta_{x2x2}+ \beta_{x3x3}$
Significance codes: ***= P <0.001
** = 0.001<P<0.01
* = 0.01<P<0.05
ns = P>0.05

of Na_2CO_3 to the molecular weight of Sargassum cristaefolium, obtained alginate molecular weight with a range of 29.83-194.93 kDa (Table 2). The highest molecular weight of alginate occurs at 60 °C, 120 min and 3% of Na_2CO_3 concentration, while the lowest alginate molecular weight occurs at 30 °C, 120 min and 5% of Na_2CO_3 concentration. The parameters of the extraction process on temperature, time and concentration of Na_2CO_3 have a positive effect on the molecular weight of alginate up to 60 °C, 120 min and 3% Na_2CO_3, decreasing at 90 °C and 5% of concentration within 60-180 min. The results of this study are consistent with the reported by Fertah et al. (2014), Hernandez-Carmona et al. (1999), and Sugiono et al. (2018). The effect of single factor extraction process of different temperature, time and Na_2CO_3 concentration significantly affected ($P<0.05$) alginate molecular weight with quadratic pattern (Figure 3). The higher the extraction process parameters, the alginate molecular weight increases, then decreases after reaching the optimal point. This can be explained by several phenomena. First, the higher the parameters process, it will cause the algae cell walls to become soft so that long chain alginates are extracted. Second, at 5% Na_2CO_3 concentration, the alginate molecular weight

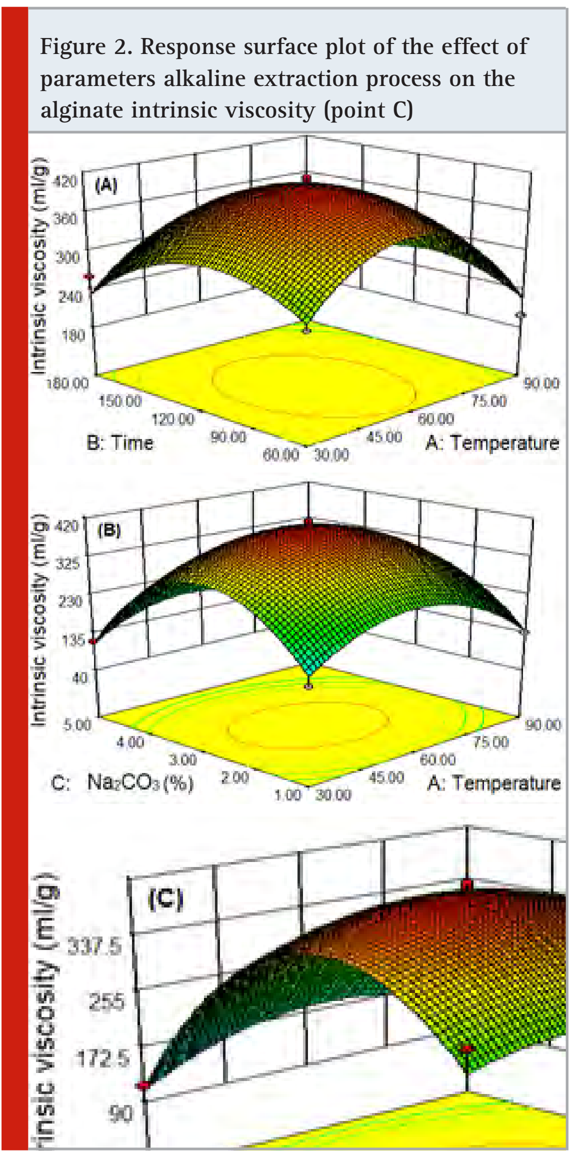


Figure 2. Response surface plot of the effect of parameters alkaline extraction process on the alginate intrinsic viscosity (point C)

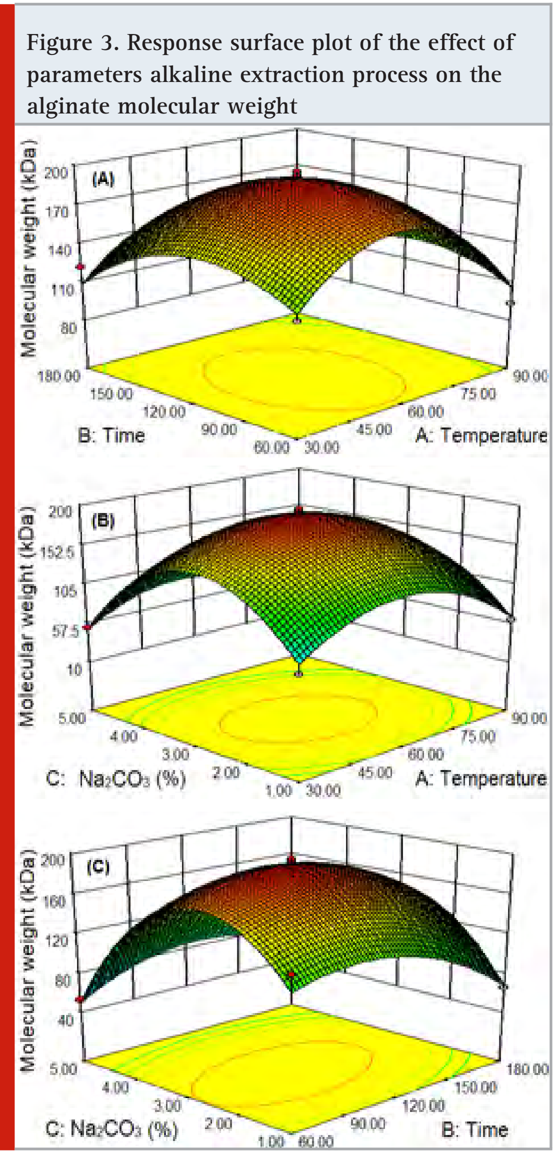
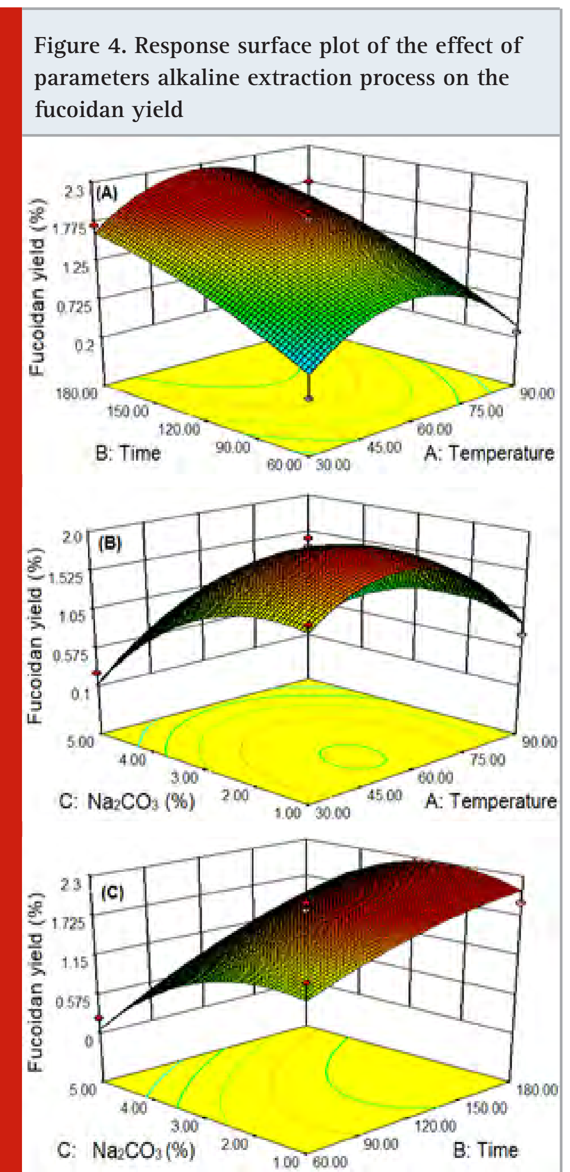


Figure 3. Response surface plot of the effect of parameters alkaline extraction process on the alginate molecular weight

is low due to the beta elimination reaction and the degradation of the alginate polymer chain (Hernandez-Carmona et al., 1999; Smidsrod et al., 1969). Third, at low concentrations of Na_2CO_3 alginate polymer, degradation occurs due to hydrolysis catalyzed by protons (Smidsrod et al., 1963). Alginate extraction from brown algae in a solution of pH 12 Na_2CO_3 obtained low molecular weight alginate as a result of the degradation of the alginate polymer chain, this is proven by the low intrinsic viscosity of alginate (Sugiono et al., 2019b).

Fucoidan yield: The results showed that the effect of different extraction parameters of temperature, time and concentration of Na_2CO_3 on the alginate and fucoidan sequential extraction processes of fucoidan yields, it is obtained fucoidan yield ranged from 0.2-1.96%. The highest fucoidan yield occurred at 60 °C, 120 minutes and 3% concentration, while the lowest fucoidan yield occurred at 90 °C, 120 minutes and 5% Na_2CO_3 concentration. The results of this study are consistent with those mentioned in the literature (Ale et al., 2012; Sugiono et al., 2014; Lorbeer et al., 2015). The different extraction process parameters (temperature, time and concentration of Na_2CO_3) had a significant effect (P<0.05) on the yield of fukoidan (Figure 4). The higher the extraction process parameters, the higher the fucoidan yield obtained, then it decreases after reaching the optimal point.

Figure 4. Response surface plot of the effect of parameters alkaline extraction process on the fucoidan yield



The results of this study are in accordance with that reported by Qiao et al. (2009), Rodriquez et al. (2010), and Lorbeer et al. (2015). The Increase of the temperature parameters and the concentration of Na_2CO_3 causes the algae cell walls to become brittle, in that case fucoidan is easily extracted. At 90 °C and Na_2CO_3 concentration 5%, fucoidan yield decreased because it was suspected that some of the fucoidan was extracted and dissolved as impurities during alginate extraction. Sugiono et al. (2019a) reported that the increasing Na_2CO_3 concentrations in the alginate extraction process would cause the algae cell wall to expand and soft, while the fucoidan is also extracted and dissolved as a impurity.

Fitting models: Box Behnken Design (BBD) from the response surface method with three central point replications used to test the effect of three variables (temperature, time and Na_2CO_3) sequential extraction of alginate and fucoidan against alginate yield, intrinsic viscosity, alginate molecular weight and fucoidan yield. The second order polynomial model of multiple responses alginate and fucoidan yield is presented in Table 3. Evaluation of the accuracy of the quadratic model of alginate yield response, intrinsic viscosity, alginate molecular weight and fucoidan yield based on parameters of model significance, correlation coefficient and lack of fit are presented in Table 3. The model compatibility

has a significance value of $P < 0.05$, $R^2 \geq 0.8$ and Lack of fit > 0.1 (Montgomery, 2005). The fitting model based on these parameters show that, the second-order of all response polynomial model is high adequate, P value in all the multiple response alginate and fucoidan yield is < P=0.05, there is no significance lack of fit because lack of fit value is more than 0.1 in all response and R^2 value more than 80% in all response.

Optimization and verification: Based on the results of the design expert version 7 program analysis, the optimal conditions for fucoidan and alginate sequential extraction occurred at 57.02 °C, 123.96 min, and 2.66% Na_2CO_3 concentration. The response of prediction value under optimal conditions is 33.93% alginate yield, 404.73 ml/g intrinsic viscosity, alginate molecular weight of 191.38 kDa and fucoidan yield 1.92% with desirability value of 0.989. The desirability value is close to 1 indicates that the predicted value of the Design Expert program has a high level of validity (Ale et al., 2012; Sugiono et al., 2019b).

The optimal process parameter conditions as predicted by the design expert program version 7 (57.02 oC, 123.96 min, Na_2CO_3 2.66%) were conducted with 3-replication verification experiments. The optimal point verification results obtained alginate yield 34.51 ± 0.87%, intrinsic viscosity 409.72 ± 7.59 ml/g, alginate molecular weight 194.08 ± 3.65 kDa and fucoidant yield 1.81 ± 0.06%. The results of verification of multiple responses alginate and fucoidan yield are in the range of predicted 95% PI high and 95% PI low. The paired t-test results found that between the predicted values of the program and the validation experiments were not significantly (P > 0.05), this indicates that the results of the validation experiments supported the results of the program analysis.

CONCLUSION

The bio-refinery of sequential alginate extraction through conventional and hydrothermal fucoidan has been succeeded to develop. This processes produces two products which are alginate and fucoidan. The temperature extraction, duration and Na_2CO_3 concentration process significantly affect to fucoidan yields and alginate multiple response. The optimal condition of temperature and Na_2CO_3 concentration of bio-refinery extraction occurs at the temperature of 57.02 °C, 124.01 min and Na_2CO_3 concentration of 2.66%, with the value of fucoidan yield is 1.81 ± 0.06%, alginate yield is 34.51 ± 0.87%, intrinsic viscosity 409.72 ± 7.59 ml/g, and alginate molecular weight of 194.08 ± 3.65 kDa.

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Conflict of Interest: The author state that in this study there were no conflict of interest.

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Occupational Stress and Job Satisfaction in Prosthodontists working in Kingdom of Saudi Arabia

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ABSTRACT

The aim of the present study was to appraise the stress levels and job satisfaction among prosthodontists working in KSA among different domains. A cross sectional survey (a type of observational study design) was conducted among prosthodontists. Information regarding prosthodontists were obtained from Saudi dental society. A questionnaire along with consent form was sent through monkey survey tool. The questionnaire was distributed into 5 domains with a total of 60 questions. Out of the 200 emails sent to the prosthodontists, 117 (58.5%) of the responses were obtained. Descriptive analysis tabulation was done by using SPSS version 22. Means and standard deviation were calculated among different domains. By applying ANOVA and comparing mean score comparison between different domain. 117 participants included in the study, of whom 86 (73.5%) were females and 31 (26.5%) were male respondents. Majority of the samples taken from participants were from age 31-40 years [54 (46.2%)] and 41-50 years [41 (35.0%)]. Most of the samples 36.8% had clinical experience between 3-8 years, and majority of them 32.5% spent more than 25 hours working in clinic per week. Most of the respondents were Saudi Nationals. 42.7% dentists expressed that 'frustration with the quality of lab work' is a reason of regret choosing prosthodontics profession as a specialty. 67.4% respondents expressed that they were passionate about the profession as it's a challenging one. 50.4% participants were of the view that they will opt for 'Prosthodontics' specialty if again given the opportunity. Overall, mean scores regarding Quality of Life (QoL) & Job Satisfaction as Prosthodontist were satisfactory. The job satisfaction and stress levels among prosthodontists working in KSA was satisfactory. However, work should be done in trying to minimize work related stress by minimizing clinical commitment better preparation of technicians which will indirectly influence job satisfaction and stress levels.

KEY WORDS: JOB SATISFACTION, STRESS, PROSTHODONTICS, DENTISTS, SAUDI ARABIA.

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INTRODUCTION

Some degree of work-related stress amongst healthcare professionals promotes competition and improves the quality of health services. However, a problem may arise when work related stress is chronic and adversely effects the physical and mental state compromising quality of life (QoL).Unfortunately, dental care professionals have known to experience high stress levels and dentistry is considered to be most stressful profession with the highest suicidal rate (Sancho and Ruiz, 2010, Pouradeli et al., 2016; Dehnad et al., 2016). A study in UK displayed that approximately 86% of dentists experienced high stress level at workplace (Kay and Lowe, 2008). Available recent evidence also suggests a probable reason for high stress amongst dentists are administrative issues, management of difficult and anxious patient, income, high travelling time, demands of work, exposure to toxic substances and long working hours, (Dehnad et al., 2016 Bhat and Nyathi, 2019). Amongst dental specialties prosthodontists and peadodontists experienced high stress levels associated to time, staff/technicians and patient (Newton et al., 2002). A recent survey on job satisfaction and stress levels among orthodontists by Alqahtani et al., (2018) proclaimed orthodontists working in Kingdom of Saudi Arabia (KSA) to be content with their job with low stress levels. Similarly, a study by Hebbal and Nair, (2012) stated that prosthodontists working in Indian institutes were found to be satisfied with teaching facilities but unsatisfied with teaching incentives and rewards. The study did not measure level of

Table 1. Demographic characteristics of the surveyed sample and their relationships to the general stress score

Variable	Categories	Frequency n (%)	General Stress Score (mean+SD)	Test value (P)
Age of the respondents	< 30 years	7 (6.0)	1.34+1.68	1.848 (0.125)
	31-40 years	54 (46.2)	1.36+0.18	
	41-50 years	41 (35.0)	2.74+1.35	
	51-60 years	11 (9.4)	2.36+1.57	
	>60 years	4 (3.4)	3.20+0.53	
Gender	Male	31 (26.5)	2.57+1.43	0.295 (0.588)
	Female	86 (73.5)	3.73+1.31	
Marital Status	Single	11 (9.4)	2.63+1.33	0.279 (0.757)
	Married	103 (88.0)	3.62+1.41	
	Divorced	3 (2.6)	2.01+1.74	
Education	Cert Prosthodontics	26 (22.2)	2.94+1.29	2.307 (0.080)
	M.Sc & Cert.	44 (37.6)	2.68+1.43	
	Prosthodontic			
	Ph.D	27 (23.1)	2.99+1.03	
	Not answered	20 (17.1)	2.99+1.03	
Workplace	Government	55 (47.0)	2.49+1.58	0.778 (0.462)
	Private	38 (32.5)	2.84+1.21	
	University	24 (20.5)	2.51+1.24	

stress and stressors among prosthodontists. To our knowledge from indexed literature, there are no studies done to evaluate the level of stress and job satisfaction amongst prosthodontics working in KSA, which makes this study unique and distinctive. Therefore, the aim of the present study was to appraise the stress levels and job satisfaction among prosthodontists working in KSA among different domains.

MATERIAL AND METHODS

A cross sectional survey (a type of observational study design) was conducted in Riyadh approved by the ethical committee of King Saud University, Riyadh KSA to evaluate level of stress among Prosthodontics. The study was in accordance to STROBE checklist for reporting cross sectional study design. The study was registered in King Saud University under ethical number (E-18-3360). The data from the respondents for this observational study were gathered in a span of three months i.e., August 2019 to October 2019. Information (email addresses) of licensed prosthodontics were obtained from the Saudi Dental Society. A link containing details of questionnaire was sent to all registered prosthodontics through survey monkey tool. A sample size of 100 participants were adequate but, since potential dropouts, non-respondents, and drop to follow up were foreseen a sample population of 200 prosthodontics were enrolled. The size of the sample was determined based on standard deviations and means scores for subscale questionnaire stated by (Wilson et al., 1998) effect size equivalent to 33% of the mean score for each subscale, with alpha=0.05 and power=0.80. Along with the questionnaire an informed consent was also mailed to the participants. The questionnaire was distributed into 5 domains with a total of 60 questions. Some of the questions were adopted and modified from a study by (Almusined et al.,2018) A group of statisticians along with subject specialists reviewed the content, did paraphrasing and modifications of the questions. A pilot study was initially performed amongst the prosthodontist to validate the components and internal consistency of the questionnaire (Cronbach's α=0.070).

The answers were computed using a four-point Likert Scale implied as, 1 strongly disagree; 2 disagree; 3 agree; 4 strongly agree. Out of the 200 emails sent to the prosthodontists, 117 (58.5%) of the responses were obtained. To minimize bias all responses were evaluated by a single investigator. Descriptive analysis tabulation was done by using SPSS version 22 (SPSS Inc., Chicago, IL, USA).

Table 2. Professional characteristics of the surveyed sample and general stress score

Variable	Categories	Frequency n (%)	General Stress score (mean+SD)	Test value (P)
Years of experience	<3 years	15 (12.8)	2.45+1.63	6.786 (0.000)
	3-8 years	43 (36.8)	2.72+1.37	
	9-15 years	27 (23.1)	2.90+1.16	
	16-25 years	17 (14.5)	2.97+0.90	
	>25 years	15 (12.8)	2.80+1.19	
Working hours / week	<25	38 (32.5)	2.76+1.15	8.096 (0.000)
	25-35	33 (28.2)	2.68+1.52	
	36-45	26 (22.2)	2.85+1.31	
	>45	20 (17.1)	2.90+0.93	
Nationality	Non-Saudi	18 (15.4)	2.65+1.44	0.591 (0.443)
	Saudi	99 (84.6)	2.37+1.14	

Means and standard deviation were calculated among different domains. By applying ANOVA and comparing mean score comparison between demographics, professional characteristics, regrets & motivations for choosing Prosthodontic.

RESULTS AND DISCUSSION

There were 117 participants included in the study, of whom 86 (73.5%) were females and 31 (26.5%) were male respondents. Table 1 presents the relationships between the general stress score and demographic characteristics of the study sample. Majority of the samples taken from participants were from age 31-40 years [54 (46.2%)] and 41-50 years [41 (35.0%)]. Around, 23.1% were Ph.D. while majority 37.6% were M.Sc & Cert. Prosthodontic. Most of the dentists were doing practice in government setup 47.0% then in private sector 32.5% and in universities 20.5%. Table 2 shows the interaction between the general stress score and respondents' professional characteristics. Most of the samples 36.8% had clinical experience between 3-8 years, and majority of them 32.5% spent more than 25 hours working in clinic per week. Of all examined professional characteristics, the general stress score was significantly correlated to working hours/week and years of experience. There were 84.6% professionals who were Saudi national then 15.4% non-Saudi nationals. 42.7% dentists expressed that 'frustration with the quality of lab work' is a reason of regret choosing prosthodontics profession as a specialty. Whereas, 30.8% dentists informed that 'physical pain i.e., back pain is a source of regret to practice prosthodontics specialty. Moreover, 25.6% expressed that 'multiple and complicated steps of prosthodontics treatment' also contribute to regret in choosing prosthodontics as a profession. However, among the factors responsible for motivations behind the choice of prosthodontics as a specialty.

35% of the prosthodontists proclaimed that 'prosthodontics ensures professional growth and better job opportunities', following by 67.4% respondents expressing that they were passionate about the profession as it's a challenging one. (Table 3). Table 4 demonstrates the respondent's expression when queried about 'which specialty would they choose if times comes again'. Among 117 respondents, 50.4% participants were of the view that they will opt for 'Prosthodontics' specialty and around 18.8% participants chose 'Orthodontics' as a specialty. This was followed by 5.1% of dentists taking 'Endodontics' as

Table 3. Respondents Regrets & Motivations for choosing Prosthodontic

What is/are the reason/s behind your regret for choosing Prosthodontics as a specialty?	Frequency	Percent
Frustration with the quality of lab work.	50	42.7
Physical pain (ex. Back pain)	36	30.8
Long working hours in the clinic compared to other specialties	28	23.9
Multiple and complicated steps of Prosthodontics treatment	30	25.6
Dealing with the same patient for a long time.	20	17.1
Relatively high cost of the materials needed to prepare a Prosthodontics clinic	16	13.7
The need for collaboration with other specialties in order to prepare a case for Prosthodontics clinic	28	23.9
I am satisfied with my profession as prosthodontist	58	49.6
What was the motivation/s behind your choice of Prosthodontics as specialty		
Prosthodontics ensures professional growth and job opportunities	41	35.0
Prosthodontics is the most prestigious specialty among other dental specialties	30	25.0
I had a passion to be a Prosthodontist because it is a challenging profession	79	67.5
My family and friend influenced me to be a Prosthodontist	4	3.4
Prosthodontists earn more and are well-paid	21	17.9

a profession. Table.5 displays analysis about quality of life & job satisfaction of prosthodontist with regards to mean score ± SD. Mean score of 3.94±1.68 was observed when inquired by prosthodontists that 'prosthodontics specialty was first choice', followed by 'facilities and resources in the clinics were adequate for delivery of dental care to patients' with general stress score 3.11+1.70. Regarding job satisfaction, mean score was higher 3.11+1.65 when asked about 'support of administrative offices, secretaries and clerical staff is adequate'. Whereas 3.05 + 1.56 score was observed when queried from the prosthodontists about 'free utilization of potentials and capabilities' and 3.15 + 1.54 score was noticed when questioned about "load of paperwork and administrative duties affect professional capabilities.

The present study provides an exclusive evaluation of prosthodontists level of stress and job satisfaction working in Kingdom of Saudi Arabia (KSA). The study stands distinctive and unique as no other work have been performed on this subject before. The overall response rate in the present study was approximately (58.5%). This low response rate was in line with a study by Newton et al., (2002) who identified source of stress among different dental specialties. In authors opinion low response rate can be attributed to extensive working hours, hectic schedule and clinical commitment among prosthodontists (Makames et al., 2012). In the current study responses were gathered through a survey monkey tool. The tool has an advantage of general usability, comprehensive feature set and security. Moreover, the tool minimizes the risk of biasness (Wright, 2006). Most of the prosthodontists in the present study were females. A strong evidence persist that female are more prone to stress compared to men as stress is psychological and gender co related (Pozos Radillo et al., 2008). Moreover, 54 (46.2%) of the respondents fall in an age range of 31 to 40 years. A trend was observed in the present study that with increase age stress levels decline. A similar drift was displayed in a study by Bhat and Nyathi, (2019).

Table 4. Respondents when asked about which specialty would you choose if times comes again

	Frequency	Percent
If you had the chance to turn back the time, which specialty would you choose		
Dental informatics	1	0.9
Endodontics	6	5.1
Law	1	0.9
Maxilofacial Surgery	2	1.7
Not a dentist	1	0.9
Operative Dentistry	1	0.9
Oral medicine	2	1.7
Orthodontics	22	18.8
Pedodontics	1	0.9
Prosthodontics	59	50.4
Public health	2	1.7
Radiologist	1	0.9
Totally out of the medical field	1	0.9
Not willing to response	17	14.5

A possible reason for this tendency is prosthodontists between age range of 31-40 years are more committed, enthusiastic and workaholic resulting in high stress levels. Whereas, with increase age they become more stable and finically secured and relaxed corelating with decreased levels of stress. These findings were found to be in concurrent with a study by Bhat and Nyathi, (2019). Furthermore, stress levels were comparatively high in married respondents compared to singles. In authors opinion since most of the respondents in the existing study were females, they have family, children to look after and work commitment resulting in increased stress levels. Though these findings were found to be in contrast with a study by (de Wet et al., 1997).

When respondents were inquired about the reasons of regret in choosing prosthodontics as specialty, 50 (42.7) claimed poor quality of lab work causes frustration. Moreover, 36 (30.8) dentists responded back pain as a source of regret. Evidence suggests back pain is the most common complaint by dentists and this trend is more prevalent in females due to lack of exercise and posture (Al-Mohrej et al., 2016)(Gaowgzeh et al., 2015).Moreover, it is a frequent cause to loss of work among practicing dental specialties

(Gaowgzeh et al., 2015). When questioned to respondents about motivation for choosing prosthodontists 79 (67.5) claimed that its challenging profession. This trend implies mature mental capacity, eagerness and well-organized and disciplined determination. The finding was in concurrent with a study by Alqahtani et al., (2018). When respondents were queried about what specialty would they choose if times comes again 50.4% responded that they will opt for prosthodontics. This reflects that most of the prosthodontists were satisfied with the profession. Work satisfaction is of utmost important. It helps a healthcare worker to perform beyond their capabilities. This indirectly results in better care of delivery and fulfilment of societal healthcare needs (Al-Hallak et al., 2018). When asked about the QoL and job satisfaction low mean scores were

Table 5. Quality of Life & Job Satisfaction as Prosthodontist

Quality of Life in Prosthodontics	Mean± SD
Prosthodontics specialty was my first Choice	3.94+1.68
My job description and responsibilities at the institution that I work at are well- defined and clear	3.50+1.58
I am satisfied with working quality of my auxiliary staff e.g. Dental Assistants, etc	3.00+1.65
I am satisfied with working quality of my Technician	2.48+1.82
Facilities and resources in the clinics are adequate for delivery of dental care to patients	3.11+1.70
My current practice situation is what I envisioned when I chose to become a Prosthodotics	2.88+1.61
I am generally satisfied with the salary/ wages and other financial benefits	3.01+1.72
I am generally satisfied with the Medical and dental treatment	2.97+1.76
services provided to me as job benefits Overall, I am satisfied with quality of life as a Prosthodontist	3.14+1.64
Job Satisfaction in Prosthodontics I am treated respectfully by the Head of my department	3.58+1.67
Support from administrative offices, secretaries and clerical staff is adequate	3.11+1.65
In general, I am treated respectfully by my senior colleagues	3.73+1.51
My organization supports professional development for improvement of their efficiency and quality of work	2.92+1.63
I am satisfied with working environment within the practice team because it is conducive and professional	3.18+1.55
I have adequate time for my professional development activities	3.03+1.60
I have adequate time for my personal and family life	2.75+1.56
I am satisfied with the practice management and care delivery system	2.82+1.57
I have good relations with my patient	3.87+1.52
My colleagues are courteous, and we enjoy working in a team	3.45+1.53
My work is recognized and appreciated by my colleagues and seniors	3.53+1.48
Patients are always on time and adhere to the appointment schedule	2.68+1.60
I feel no problem while communicating with staff	3.59+1.49
I feel no problem while communicating with my patients	3.38+1.68
The load of paperwork and administrative duties affect my professional capabilities	3.15+1.54
Patients' unrealistic expectations burn me out	2.93+1.64
Amount of workload is too much and killing	2.75+1.64
I face too much pressure from my seniors	2.51+1.82
I can freely utilize my potentials and capabilities	3.05+1.56
I have a liberty to choose appropriate working methods and materials	3.30+1.58
Overall, I am satisfied with my job as a Prosthodontist	3.71+1.59

observed in showing dissatisfaction with working quality of technicians (2.48±1.82) and medical and dental treatment services as job benefits (2.97±1.76). Moreover, inadequate time with family (2.75+1.56), non-adherence to appointment schedule by patients (2.68+1.60), hectic clinical hours (2.75+1.64), and pressure by seniors adds to poor job satisfaction (2.51+1.82). Encouraging, conducive environment by peers and seniors plays a vital role in reducing occupational stress and improving work performance. These factors contribute and plays a huge role in success and delivery of care services (Khalighi et al., 2018). Non-adherence to appointment schedule as a cause of job satisfaction, stress and anxiety in dentists was also observed in a study by (Rada and Johnson-Leong, 2004). Furthermore, family support, motivation and pleasant family life reduces work related stress and burnout syndrome among dentists. But unfortunately, prosthodontist working in KSA explained that due to clinical commitments and non-flexible working hours they didn't get enough time with family.

From the results of the present study it can be determined that though stress levels among prosthodontists working in KSA were nominal. The mean and SD scores give an indication that prosthodontists were not happy with hectic commitments and demanding prosthetic work. However, overall job satisfaction among prosthodontist were satisfactory. The study has limitations based on small sample size. A qualitative design study will be more appropriate to cater the responses of prosthodontist giving better reasons of job dissatisfaction and causes of stressors. For future studies, a comparison between Saudis and Non-Saudis job satisfaction and levels of stress as prosthodontists should be also performed

CONCLUSION

The job satisfaction and stress levels among prosthodontists working in KSA was satisfactory. However, work should be done in trying to minimize work related stress by minimizing clinical commitment better preparation of technicians which will indirectly influence job satisfaction and stress levels.

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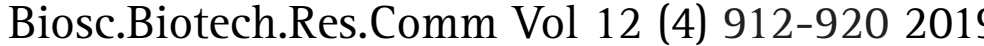
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Effect of Mastery Cooperative Studying and Individual Inquiry on Attaining the Technical Aspects of the Stages of Shot Put and Digital Achievement Using the Linear Procedure Among Vietnam University Students

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ABSTRACT

Physiological indicators say a lot about the preparedness and performance of students. This study investigates the influence of mastery cooperative studying generalship and individual inquiry in using the linear procedure among female students of An Giang University,the study sample consisted of (57) female students enrolled in track and field course (1) at the Deparment of physical education at An Giang University during the first quarter. The sample was divided into three equal groups. The first group (n=19) taught using the mastery cooperative studying generalship, the second group (n=19) taught using the individual inquiry generalship, while the third group (n=19) was taught using the traditional procedure. Results showed that there were statistical differences in favor of the mastery cooperative studying generalship and individual inquiry generalship over the traditional way.

KEY WORDS: MASTERY STUDYING; INDIVIDUAL INQUIRY; SHOT PUT; LINEAR WAY.

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INTRODUCTION

The mastery cooperative studying and what accompanying of sequentially procedural steps and organized prepare the students for individual work and the division of students into heterogeneous groups to work together using dialogue and discussion and exchange of information and classification and interpretation and analysis, and evaluate and make comparisons and draw conclusions and generate ideas and guidance from the teacher; important in highlighting the role of student and refine her personality and the ability to cooperate and communicate with others and acceptance and access for students to the point of perfection in the acquisition of skills and motor abilities (Walid Hammouri et al, 2016). Strategies and teaching procedures have evolved as a result of the inevitable evolution of contemporary democratic societies based on modern educational psychology and educational research that took on the increase, because of the awareness of teachers and their need to change the traditional teaching procedures in education process (Mosston & Ashworth, 1986).

And the creation of modern teaching strategies to help students achieve their objectives through the creation of procedures of teaching alternative consistent with the scientific development and technological progress, which has made the world a small village; which worked to increase the acceleration of knowledge transmission rapidly surpassing distances and overcome as soon as possible and with less effort than the global openness easy and follow-up all new and sophisticated (Walid Hammouri et al, 2016). Many studies agree that cooperative studying has many benefits that distinguish it from the rest of the strategies through the development of the capacity of students to creative thinking and thinking skills over knowledge and capabilities of different thinking and take advantage of learners from each other's experiences and improve learners' attitudes towards themselves and others and curriculum, teacher and educational institution, Carol suggested a model for mastery of studying which is based on three concepts.

1. The ability to understand (Understanding), it means learner's ability to absorb and understand the nature of the educational mission and understand it,and try to decode and analyze their symbols and try to integrate them in the proceeds of knowledge in order to mastery.

2. The concept of educational mission (studying task) associated with the success of the learner in the amount of time it takes to learn that task, or how much they actually takes the learner to learn and interact with the task.
3. The quality of teaching (instructional Qualities), this concept confirms to follow a variety procedures of teaching providing content to suit the majority of students, from these procedures that have extreme importance in Carol model for mastery studying is cooperative studying procedure. The mastery studying from the teaching strategies, which must be arranged and sequenced on the pyramid, to be taught segmented into clear phases of studying in an educational skill or more than , then testing students after they finished, if they do not reach to the Mastering, they must provide additional time to performMastering in testing, and control of the main phases of skill before moving on to the other skills (Mosston & Ashworth,1986).

As for the generalship of directed individual inquiry, it is a way to search and investigate individually that means every individual looking for the answer to a specific question and research and investigation to find the best ways and the shortest to explore the situation or a problem concern to all students, then determined this situation and the problem and the questions that will discuss for an answer to it, then everybody is integrated in the activity that is being sought during the discussion of the results that have been reached between the students them self, and the teacher on the other hand. The research results examines the effectiveness of maturing collaborative studying and personal studying strategies in achieving the technical aspects of digital shooting and achievement stages by linear procedure among students of An Giang University, the survey is a more modern teaching generalship that is effective in developing scientific thinking in students, because it creates opportunities for students to practice teaching

procedures and practice surveys. about their own skills, so students will think and search for their teaching results, the survey generalship depends on establishing the processes applied on the basis of facing student problems, to give him the opportunity to create independent thoughts and demand the implementation of design procedures To consider achieving solutions and implementation, students collect data and sort it, then reach conclusions under the supervision of teachers.

MATERIAL AND METHODS

This study investigates the influence of mastery cooperative studying generalship and individual inquiry in using the linear procedure among students of An Giang University, the study sample consisted of (57) under female students who were physical education at An Giang University. (M age = 19.6 sd =1.33) (Chanh Thuc Dao, 2018). All the subjects were enrolled in track and field course (1), they were divided deliberate into three groups, the first group (n=19) taught by mastery cooperative studying generalship, the second group (n=19) have been using their individual oriented inquiry in teaching, while the third group (n=19) taught by the traditional way in teaching, Permission to conduct this study was received from the study sample and head dean of faculty of physical education at An Giang University. The students were told the purpose of the study and their rights as participants and they were asked to sign a consent form. The evaluation test of technical phases of the shot put skill was designed by the author after consulting

Table 1. The average and standard deviation for the applied skills of shot-putting and its pre and post distances for the three groups

Skill parts	Group	Pre measurement		Post measurement	
		Average	Deviation	Average	Deviation
Preparation	Traditional	2.24	0.44	2.87	0.23
	Perfected cooperative	2.13	0.66	8.13	0.72
	Oriented survey	2.43	0.48	8.51	0.79
Crawling	Traditional	2.21	0.34	2.76	0.30
	Perfected cooperative	2.19	0.42	7.81	0.11
	Oriented survey	2.56	0.74	8.03	0.79
Power situation	Traditional	2.37	0.22	2.83	0.42
	Perfected cooperative	1.99	0.56	7.73	0.78
	Oriented survey	2.66	0.60	8.02	0.67
Basic accelerating	Traditional	2.52	0.42	291	0.42
situation	Perfected cooperative	2.42	0.51	796	0.28
	Oriented survey	2.46	0.58	829	1.03
Hand motion situation	Traditional	2.31	0.81	297	0.29
	Perfected cooperative	2.46	0.53	833	0.09
	Oriented survey	2.81	0.67	929	1.07
Covering	Traditional	2.31	0.39	306	0.27
	Perfected cooperative	2.56	0.38	807	1.08
	Oriented survey	2.72	0.32	844	0.75
Distance of Shot- put	Traditional	5.53	0.59	606	1.01
	Perfected cooperative	5.19	0.42	713	0.21
	Oriented survey	5.52	0.46	840	0.34

Table 2. The average, standard deviation and the calculated (t) result between the pre and post measurements for the perfected cooperative generalship group

Skill Parts	Measurements	Average	Standard deviation	t_{result}	P
Preparation	Pre	2.32	0.67	24.79	*0.000
	Post	8.11	0.71		
Crawling	Pre	2.12	0.44	24.13	*0.000
	Post	7.68	0.91		
Power situation	Pre	1.83	0.62	18.54	*0.000
	Post	7.39	0.95		
Basic accelerating situation	Pre	2.05	0.56	25.06	*0.000
	Post	7.85	0.87		
Hand motion situation	Pre	2.26	0.56	27.87	*0.000
	Post	8.31	1.02		
Covering	Pre	2.72	0.21	18.76	*0.000
	Post	8.02	1.18		
Distance of Shot- put	Pre	5.61	0.38	18.03	*0.000
	Post	7.09	0.29		

Table 3. The average, standard deviation and the calculated (t) result between the pre and post measurements for the traditional group

Skill Parts	Measurements	Average	Standard deviation	t_{result}	P
Preparation	Pre	2.22	0.57	14.19	*0.000
	Post	2.91	0.61		
Crawling	Pre	2.32	0.44	11.13	*0.000
	Post	2.68	0.31		
Power situation	Pre	2.21	0.62	9.54	*0.000
	Post	2.32	0.55		
Basic accelerating situation	Pre	2.25	0.52	15.06	*0.000
	Post	2.88	0.37		
Hand motion situation	Pre	2.23	0.46	17.37	*0.000
	Post	2.31	0.22		
Covering	Pre	2.72	0.21	14.56	*0.000
	Post	2.02	0.18		
Distance of Shot- put	Pre	2.60	0.28	11.03	*0.000
	Post	2.09	0.21		

scientific references of track and field federation and international coaches, the author designed an educational program based on the mastery cooperative studying generalship and another one based on individual- oriented in order to improve the performance of the shot put skill for the study sample, pre-test was measured to the three study samples for the variables of this study .the variables included the stages of the shot put and digital achievement after a warm-up ,the author recording the best of three tries, according to international law to the shot put using the legal tools approved by the International Association of track and field.. After applied the two months educational program which contained (10) units, twice units a week each unit was (90) minutes of training, the post-test were taken, The statistical processing included arithmetic mean, standard deviation, Analysis of variance, REGW comparisons Posterior.

RESULTS

Table (.2) shows the average and the standard deviation and the calculated (t) result between the pre and post measurements for the perfected cooperative studying students. (t) result shows the difference for the static significant in the applied skills of shot-put and its distance, in which all the calculated (t) result were higher than 2.878 (TM 2.878) for the level 0.01, where these differences in favor to the post measurement. Table (3) shows the result average and standard deviation of the calculated (t) result between the pre and post measurements for the traditional studying group, the calculated (t) result shows the difference for the static significant in the applied skills of shot-put as well as its distance, in which all the results were higher than 2.878 (T M 2.878) for the level 0.01, where these differences in favor to the pre measurement. (Chanh Thuc Dao, 2018). Table (4) shows the average and the standard deviation and the calculated (t) result between the pre and post measurements for the individual oriented survey group, the calculated (t) result shows the difference for the static significant in the applied skills of shot-put as well as its distance were higher than 2.878 (TM 2.878) for the level 0.01, where these differences in favor of the post measurement. (Chanh Thuc Dao, 2018).

Table (5) shows the results for analyzing the contrast unilateral for the stages of shot-put skill and the throwing distance in the pre measurement as well as the calculated (F) result which shows the difference for the static significant for the level 0.01 in the applied skills of shot-put and its distance, in which all the calculated results of (F) results were higher than 3.78 (FM 3.78). Table (6) shows the results for the REGW test for choosing the contrast source in the applied skills of shot-put and its distance. The results found that the difference between the traditional group and the perfected cooperative group and between the traditional group and singles oriented survey group, while there wasn't any difference with a static significant between the perfected cooperative group and the singles-oriented survey group in the applied skills or the distance of shot-put.

DISCUSSION

The results of this study was a positive influence in using the perfected cooperated studying generalship on the study variables in acquiring the technical stages for effective performance of shot-put in a linear way. There was a static significant in improving those variables. Attribute the author that the positive influence to the effectiveness of teaching generalship perfected cooperative studying and characterized by positive interaction and investment times of lectures fully effective and take into account individual differences among students on the one hand and the teacher on the other hand, and that can work on the access of all students to a high degree of workmanship skills for all technical stages of performance and enable students of the ability to The ability to make decisions for themselves after providing them with the necessary and required information after explaining the technical stages of the event, presentation to parts of effectiveness during the lesson, which led to increased digital achievement to the performance of the students and it is due to mastering the technical stages and their characteristics by taking into account individual differences among students and competition among them and help each other and perseverance in order to increase the degree workmanship and distinguish them from each

other and their interaction outstanding with this procedure and the diversity of teaching procedures in achieving the objectives and so that the procedure of the effective role and influential in the educational process in the curriculum to be implemented and that the teaching procedures affect studying and the degree of saturation speed in studying and adaptation with the way depends on the proper understanding of, and the desired state of studying, and research and investigation. This will be the role of the student to take responsibility by harnessing all the educational possibilities, educational techniques and modern teaching procedures and indirect procedures of assessment, and these results are consistent with the findings of both studies (Rayyan, 2005). Researchers find that the traditional way had an influence on the acquisition of the technical stages in the shotput and digital achievement in the dimensional measurements, but the results of the REGW test showed that the differences were in favor of the perfected cooperative group over the traditional group This result is in agreement with the studies of (Mohammad,2000).

Woods (2002) points out that those play an important role in triggering students' creativity as they represent the field of experts who are to judge the creative output. They introduce a shift in pedagogy, moving towards an inclusive approach, where the environment is permissive and safe and learners are in control of their learning process. Teachers should allow co-construction of knowledge, being reflective practitioners, supporters and facilitators and not bureaucrats, nor technicians applying governmental policies without questioning them or inhibitors by being overly didactic or prescriptive. (Ferrari et al., 2009) argue that students should not be considered merely receivers of information, on the contrary, it is important that they assume the role of discoverers, but support and guidance are needed in order for them to succeed. This can be enhanced when students are exposed to e-learning where electronic devices are used enabling them to control their learning. For this, teachers need to be prepared both on the pedagogical side, being aware of the means and ways to foster autonomy and student-centeredness and on

Table 4. The average, standard deviation and) calculated (t) result between the pre and post measurements individual oriented survey group

Skill Parts	Measurements	Average	Standard deviation	t_{result}	P
Preparation	Pre	2.65	0.49	36.09	*0.000
	Post	7.67	0.85		
Crawling	Pre	2.21	0.69	27.23	*0.000
	Post	6.98	0.54		
Power situation	Pre	2.57	0.43	34.12	*0.000
	Post	7.89	0.65		
Basic accelerating situation	Pre	2.31	0.39	25.91	*0.000
	Post	8.19	0.99		
Hand motion situation	Pre	2.65	0.51	19.06	*0.000
	Post	8.76	0.95		
Covering	Pre	2.72	0.59	28.45	*0.000
	Post	8.12	1.04		
Distance of Shot- put	Pre	5.45	0.42	12.23	*0.000
	Post	7.01	0.60		

the subject-knowledge. According to the REGW test results for choosing the contrast source in the applied skills of shot-put and its distance. We found that the difference were between the traditional group in one hand and the perfected cooperative group and singles oriented survey group in the other hand. Otherwise there wasn't any difference with a static significant between the two groups; perfected cooperative and the singles-oriented survey, in the applied skills or the distance of shot- put, these results are consistent with a study (Haidan& Ali 2010).

The result found a positive and special influence of the oriented survey stratify on the study variables in acquiring the technical stages for the effective performance of shot-put in a hasty manner. It provided positive interaction and it was a good investment of lectures time, taking in consideration individual differences between students through discussion and dialogue which held between the students and the teacher the thing that helps them solve the prefomance problems, Hassan (1989) noted that through discussion and dialogue which held between the students and the teacher, they investigated the most appropriate way for the completion and criticism of colleagues and sensitivity to detect problems and solve them in a scientific logical way, leading them to be creative in discovering the procedures and technical ways to check the level of advanced studying, guiding them to

Table 5. Results for analyzing the contrast unilateral for the stages of shot-put skill and the throwing distance in the post measurement for the three groups

Skill Parts	Contrast Source	Sum of freedom	Degrees of freedom	Average squares	F result	P
Preparation	Between groups	272.12	2	132.12	289.12	*0.000
	In groups Total	19.01	291.13	55	57	0.42
Crawling	Between groups	223.49	2	118.72	178.39	*0.000
	In groups Total	23.09	246.58	55	57	0.34
Power situation	Between groups In groups	251.71	2	119.56	182.13	*0.000
	Total	24.36	276.07	55	57	0.67
Basic accelerating situation	Between groups	276.34	2	132.23	201.09	*0.000
	In groups Total	29.72	306.06	55	57	056
Hand motion situation	Between groups	253.23	2	123.56	178.65	*0.000
	In groups Total	26.32	279.55	55	57	0.65
Covering	Between groups	243.45	2	125.56	181.09	*0.000
	In groups Total	27.12	270.57	55	57	0.71
Distance of Shot- put	Between groups	14.27	2	9.08	45.56	*0.000
	In groups Total	24.65	38.92	55	57	0.15

acquire scientific thinking and analytical and critical skills of the positions and the various educational missions, they had assigned to the various students who work with each other and debating among themselves, so each individual felt responsibility towards her group in order to develop the right direction towards the study and the development of skills and the ability to generate ideas to get to the good performance and constructive and valuable information to achieve their goals, the cooperative studying generalship is one of the strategies that needs to be art of leadership to reach the goals, and it is one of the influential strategies in the educational experiences, which calls for attention in the teaching-studying process, and the thrill and attention and motivation to learn, and that the students who are working in accordance with cooperative groups can understand and accommodate educational materials, better than students who are working individually and these results are consistent with studies and (Mohammad et al, 2016). And the other studies in the field of science survey showed the effectiveness of the generalship, such as (Al-Zghool & Al-Mahameed, 2007). The current study and the results that have been reached, it is concluded the following:

Table 6. Shows the results for the REGW test for choosing the contrast source in the applied skills of shot-put and its distance. Through

Skill parts	Students	Average	Deviation	Perfected cooperative	Oriented survey
Preparation	Traditional	2.97	0.31	-5.31	-5.69 -0.39
	Perfected cooperative	8.12	0.67		
	Oriented survey	8.58	0.88		
Crawling	Traditional	2.19	0.26	-4.91	-5.09
					-0.12
	Perfected cooperative	7.68	1.02		
	Oriented survey	7.92	0.87		
Power situation	Traditional	2.78	0.39	-4.81	-5.09
					-0.27
	Perfected cooperative	7.17	0.97		
	Oriented survey	8.01	0.78		
Basic accelerating situation	Traditional Perfected cooperative	2.84 7.94	0.34 0.88	-5.03	-5.29 -0.30
Hand motion situation	Oriented survey Traditional	8.29 2.97	1.01 0.21	-5.36	-5.67 -0.29
	Perfected cooperative	8.33	1.04		
	Oriented survey	8.68	1.01		
Covering	Traditional	2.98	0.19	-5.09	-5.33
					-0.34
	Perfected cooperative	8.02	1.21		
	Oriented survey	6.02	0.56		
Distance of	Traditional	7.19	0.29	-1.06	-1.32
Shot- put					-0.27
	Perfected cooperative	6.09	0.31		
	Oriented survey	7.46	0.59		

+ The process of using the perfected cooperative studying generalship accelerate studying and the acquisition of motor skills and improve the level of technical performance of the stages of shot- put . The using individual survey directed generalship accelerates the studying process and improve the level of technical performance and achievement of the effectiveness of the distance achieved with the shotput.

+ The using perfected cooperative studying generalship helps achieve distance in the shot-put games for the study sample. Saves a lot of time and effort and the ability to acquire practical skills among the sample of the study; Raises the students thinking and accepting teamwork among students of cooperation and help each other and overcome them in all educational studying situations in groups.

+ The using individual survey orientated generalship raises the students thinking and acceptance of individual work between students by researching and investigating to find the best solution to solve the problems and to answer questions posed to students by the teacher.

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Comparative Microbial Assessment of Local and Commercial *Spirulina platessis* Samples

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ABSTRACT

Present study is concerning with the assessment of putative bacterial contamination in *Spirulina*, to assert the further studies and pose strict monitoring to get pure supplements. Our study, aim was to assess any bacterial and molds contamination of local and commercial "*Spirulina*" supplements available in Jeddah region market. During the study, cyanobacteria "*Spirulina*" was dried and commercially available *Spirulina* were subjected to serial dilution 10-1, 10-2, 10-3 and 10-4 and spread on media. The result revealed that only 10-1 produced colonies on culture Plates. In addition, when they were cultured on specified media e.g., chocolate blood agar, Eosin methylene blue, MacConkey agar, Mannitol salt agar, Sabouraud Dextrose Agar and Salmonella-Shigella agar no pathogenic bacterial colony was found. This study concludes that both local and international *Spirulina* supplement is clear from any pathogenic bacteria and mold.

KEY WORDS: *SPIRULINA*, MICROBIOLOGICAL ANALYSIS, PATHOGENIC MICROORGANISMS.

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INTRODUCTION

Raw materials in biological system can be contaminated with an excessive number of bacteria, fungi and algae due to the medium conditions in which they grow. Today, wastewater treatments needs to filter out the nutrients such as phosphorus and nitrogen that could be hazardous to the environment if not removed from water. The phosphorus and nitrogen contaminations lead to severe hazard to ecosystem if water containg these contaminants mingle into the rivers and stream. Now a days, heavy metals contamination became a global issue due to their high toxicity, non-biodegradability and carcinogenicity. The heavy metals are added up in food chain and get accumulated in human tissues and cause toxicity and carcinogenicity. Early researches conducted on heavy metals removal have shown the inactive biomass play an essential role on this process including biological growth conditions. A filamentous cyanobacterium including bacteria and fungi have the potential to fix such these contaminated materials. (Davis et al., 2003, Gao et al., 2009, Ruiz-Marin et al., 2010, Sud et al., 2008).

Nowadays, 800 million of people all over the world are suffering from malnutrition; 200 million are children and this cause a serious problem in public health (Anon, 2006). In the 21st century, depending on local natural sources particularly microalgae is the best food for future, greatly valuable for bioactive compounds and relatively cheap. The most important genus is *Spirulina* (Arthrospira), which has a considerable micro and macronutrients content (Soni et al., 2017), rich in protein, betacarotene, vitamins and minerals (Ores et al., 2016, Matos et al., 2017). Previous works on *Spirulina* have shown that this microalgae mainly s. platensis can improve the immunity system of organism and promote calcium absorption (Shabana et al., 2017). Moreover, analysis of s. platensis from various product have shown that it's contain up to 70 % protein, carbohydrates, essential amino acids, essential fatty acids such as palmitic acid, linolenic acid and linoleic acid (Lupatini et al., 2016), pigments like chlorophyll a, phycocyanin, carotenes and some minerals. As well as, it has a huge health benefits as antibacterial and antiviral activities, antioxidant, antiinflammatory, anticancer, also it works against obesity, malnutrition, diabetes, anemia and heavy metal toxicity, it has been used as feed additives in many animal species and in waste water treatment (Matos et al., 2017). The genus *Spirulina* is a planktonic photosynthetic cyanobacterium can also fix CO2 therefore reducing carbon dioxide emission (Meng et al., 2009), it was also shown that *Spirulina* seems to be a safe food as shown by Al-Dhabi 2013.

Recently, industrial countries for example China considered as the biggest *Spirulina* manufacturer all over the world (Li et al., 1997), due to an incredible achievements this kind of industry has more attention in China and increased rapidly. The International Energy Agency (IEA) declared that over 50 % of the worldwide of 10,000 tons annually is produced in China (Huo et la., 2011), which generates about $570 million (Yun-Ming et al., 2011).In addition, different organization such as the United Nations World, World Health Organization and UNESCO confirmed that the microalgae S. platensis is the most valuable food for tomorrow. On the other hand, NASA and

Table 1. Total aerobic viable count

Dilution	24 H CFUs/ml		48H CFUs/ml		EU standard	WHO
	Commercial sample	Local sample	Commercial sample	**Local sample**		
1:10	11±0.5	8 ±0.5	10 ±1	8 ±1		
1100	0	0	0	0	1.0 x10^5	1.0 x10^5
1:1000	0	0	0	0		
1:10000	0	0	0	0		

European Space Agency used it in long-term space mission in space.

MATERIALS AND METHODS

Preparation of water extracts of *Spirulina:* Samples were collected from internet distributors in Jeddah region, Saudi Arabia in 2019 (national and commercial powders). Based on the product label of the commercial sample the manufacturing company: California Gold Nutrition and the original country is India. For the sample preparation, 10 g was emulsified with that of 90 ml peptone H2O. The samples were frequently agitated in water bath for 15 min at 45 °C.

Microbiological analysis: Colony forming method was used for the assessment of microbial load. For this reason, the samples were diluted with distilled water. For dilution, One ml of sample was transfer to 9 ml of distilled water. Furthermore serially diluted with that of 9 ml distilled water. Then these diluted samples were transfer to total plate agar dishes. The plates were incubated for 48 hours at 35±2°C to determine the total plate counts for bacteria. In addition, a numbers of different selective media were used to detect pathogenic bacteria (APHA, 2005). Determination of pathogenic microorganisms: Samples were serially diluted and then transfer to blood agar plates. The plates were incubated for 24 hours at 35±2°C. After incubation, count the colonies on each of the plates and make a subculture the growth from blood agar to different selective media (ATCC, 1984).

RESULTS AND DISCUSSIONS

A comparative analysis of commercially available *Spirulina* and locally collected *Spirulina* were carried out. The *Spirulina* sample collected locally from Jeddah region was processed and crushed to powder. The commercially available and locally collected *Spirulina* powder were dissolved in deionized distilled water to get a homogenized mixture. The mixtures were subjected to serial dilution e.g., 10-1, 10-2, 10-3, 10-4. The results (Table 1) revealed that each dilution when spread on plate produced different number of bacterial colonies after 24 h and 48 h of incubation. In addition, 10-1 dilution showed CFU/mL but all the other dilution such as 10-2, 10-3 and 10-4 showed no bacterial colonies.

Growth on different media: Different specified media such as chocolate blood agar, Eosin methylene blue, MacConkey agar, Mannitol salt agar, Sabouraud Dextrose Agar and *Salmonella-Shigella* agar were used to spread the 10-1 dilution of both collected and commercial *Spirulina* powder. The results (Table 2) showed that no colonies were grown in any of the above media. The absence of the colonies on any specified media revealed that there were no pathogenic bacterial or fungi present in the food such as Salmonella and Shigella. EU, European Union Pharmacopeia (Kneifel et al. 2002), WHO, World Health Organization (Belay, 2008). *Spirulina* refers to nutrition rich, cyanobacterial biomass that can be consumed by humans. The *Spirulina* has been investigated be very rich Proteinous supplement. The biomass of *Spirulina* is used

Table 2. Determination of pathogenic organisms

Selective media	Observations		EU standard	WHO
	Commercial sample	Local sample		
chocolate blood agar	No growth	No growth	No data	No data
Eosin methylene blue	No growth	No growth	No data	No data
MacConkey agar	No growth	No growth	No data	No data
Mannitol salt agar	No growth	No growth	No data	No data
Sabouraud Dextrose Agar	No growth	No growth	1.0 x104	1.0 x103
Salmonella-Shigella agar	No growth	No growth	Absent in 10g	Negative

interesting element to overcome the malnutrition especially in the countries where malnutrition and deficiencies in nutritional cause serious problems (Wu and Pond. 1981). It has been an ancient traditional food in many parts of the world and is remain a great source to overcome malnutrition as well as undernutrition in all over the world. In the present study, analysis of *Spirulina* local and commercial was conducted to assess the contamination in both samples. The presence of total bacterial micro-flora was resulted in local and commercial *Spirulina* in the dilution 10-1 only. However, 10-2, 10-3 and 10-4 dilution showed no colonies of the bacteria. The colonies in 10-1 dilution could be due to the violation of hygienic condition at the time of harvest. Unlike present results, other studies conducted on the importance of shelf life and packaging as well as nutritional values showed a significantly high concentration of bacteria which was assumed to be contamination of *Spirulina* samples (Tidjani et al., 2013 Laurencia et al. 2017).

According to the study of Karar et al. (2016), the absence of faecal coliform was due to the improve quality of the samples used in serial dilution followed by culture. Final out comes of the microbiological study should be according to the national and international standards (Belay, 2008; Dillon and Phan, 1993). Optimized standards such as coliform densities and standard plate count are being used to counter mal handling in food industries during food processing (FDA, 1998).In addition, when collected and commercial *Spirulina* suspensions were spread on different media e.g., MacConkey agar, Eosin methylene blue, chocolate blood agar, Mannitol salt agar, Sabouraud Dextrose Agar and *Salmonella-Shigella* no bacterial colonies were found. These results were comparable with the studies conducted on commercially produced Spirulina, which proved absence of pathogenic bacteria e.g., *Shigellae-Salmonella*, or Staphylococci in the *Spirulina* samples (Tidjani et al., 2013).

Some of the studies also show that fungi did not grow in *Spirulina* supplemented samples, and this is possibly due to the high alkalinity the culture medium is an excellent barrier against bacteria, fungi, or algae (Kumar et al., 2011, Butler and Day., 1998).Vermorel et al. (1975) performed a study on microbes associated with *Spirulina* and concluded that presence of the microbes were very rare or non-pathogenic in *Spirulina*. Moreover, alkaline pH of the growth media were also a factor to stop the microbial growth (Chapeland-Leclerc et al., 2005). In short, the *Spirulina* collected locally or commercially were almost free of pathogenic bacteria and could be used as food supplement. It is well known that, food industry required standard methods such as; bacterial plate counts to monitor the food products during processing (FDA, 1998). These methods differed from country to another in a small range). Plate counts of aerobic A. platensis in Sweden, France, California and Japan have been reported (Costa et al. 2003; Vonshak 1997).

Previous work by (Yousef et al., 2014) found that the total bacterial counts for spirulina platensis in closed and open system was between 1.2 x103- 1.4 x104 respectively. Vonshak (1997) as well reported that limits of the standard plate count for *Spirulina* in dry form are < 0.05x106 cfu g-1 in Japan, < 10x106 cfu g-1 in Sweden, < 0.1x106 cfu g-1 in France and < 1 x 106 cfu g in USA. In addition, it has been reported that the level of contamination would be decreased by drying process. Indeed, all obtained data shown in (table 1, 2) regarding the microbial contents were satisfactory according to the European Union and World Health Organization standards.

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Contrast Behavior by Female Dragonfly, Orthetrum sabina While Mating With its Territorial Male

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ABSTRACT

There is variation in receptiveness of female dragonfly to male waiting in territorial ponds. The present study reported how a female of *Orthetrum sabina* agreed or not for mating with a male in different situations. In usual or first case after grasping female by male, female helped him for flying at perch and to make copulatory wheel the 'tandem'. But in the second case, a non receptive female denied territorial male for making tandem was also noted. It was observed that non receptive female was evasive to make tandem and appeared to be resisting the males grasp by her wing vibration. She got clung to male's abdomen remained inactive for 7 minutes in motionless pose and appeared like dead. Although she injured her wings by vibrating she tried to escape and got freed from the grasp of male without copulation. One failure of copulation added to account of that territorial male was seen. We conclude that in second case the female may be immature and not ready for mating, or the male was not of her choice, may be the eggs deposition by her would have already been accomplished for the day or female may already have carried the sperms so she rejected that territory owner. The significance of this study is to understand different ways for breeding behavior in selected species to save their future.

KEY WORDS: *ORTHETRUM SABINA*, MATING, TANDEM, NONRECEPTIVE FEMALE.

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INTRODUCTION

Behavior is a way that an animal interacts with other animals and express themselves. Some stimuli are responsible for change in behavior that may be external or internal. Females usually make a larger investment in reproduction than males (Bateman, 1948). Reproductive effort is vital to life-history theory (Roff, 1992). Recently it has been realized that a female's reproductive effort may be influenced by the quality of her partner (Burley, 1986); (Eberhard, 1996). Multiple mating by males is well known in insects, counting the Lepidoptera. A male's reproductive yield is closely associated with the number of females he is able to inseminate, so it is broadly accepted that the best male strategy to maximize fitness is usually to obtain as many mates as possible (Trivers, 1972); (Thornhill and Alcock 1983). Females getting small spermatophores often reduce their own reproductive output, exercising cryptic female choice through differential allocation that depends on the excellence of her mate (Wedell, 1996).

The exclusive mating morphology of the Odonata makes it unfeasible for a male to copulate without assistance of the female. Before a male can mate, he must take a female in tandem by clutching her with his anal appendages. Once in tandem and later than a male has transferred sperm to his penis vesicle on his second abdominal segment, a female must elevate her abdomen and should be willing to engage her genitalia with the male's (Waage, 1984). Males have to perceive a female receptivity to focus efforts in potentially successful interactions (Bonduriansky, 2001), since courting a non-receptive female would entail a waste of time and energy (Hoefler, 2008, Hadley 2019). In dragonfly oviposition sites may overlap with mating sites for most of the dragonfly (Corbet, 1962). The anticipation of an adult dragonfly is to find suitable habitat for mating and oviposition. Strong male can be describing with the health of territory he having. There is overwhelming competition to get healthy territory as most of the receptive females generally visits there for reproduction. The female that carried mature eggs can be define as 'receptive', that have choice for mating or not depending on the costs and benefits to her of doing so. Lacking mature eggs and linearly resists mating are 'unreceptive female. Hypothesis by (Emlen & Oring, 1977) acquaint that sexual selection on males increases as males are increasingly able to monopolize females and critical resources. This hypothesis only addresses male fitness and male mating patterns; female aspirations are not even considered as a source of breakaway in male reproductive success. Despite increasing documentation that females of many species exercise considerable control over reproductive decisions (reviewed by Andersson, 1994), and indications from genetic models that mate choice is feasible e.g. (Kirkpatrick, 1982); (Pomiankowski, 1988); (Price, Schluter & Heckman, 1993). Collaboration between the sexes in evidently showing their sexual intentions is common. Most females are receptive for mating only on days when they have mature eggs to lay, and become unreceptive after laying an egg clutch (Fincke, 1986a). Female Calopteryx are able to reject courting males, and this raises the question why males simply do not capture ovipositing females forcibly in tandem.

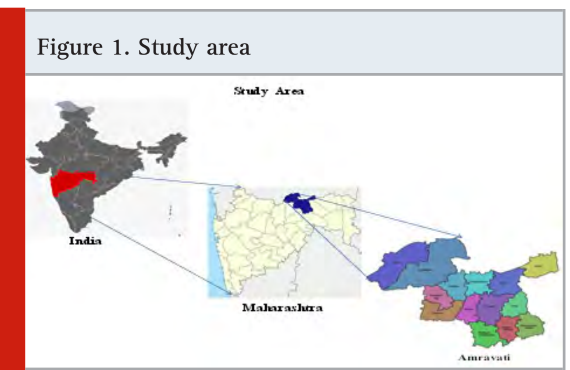
Because the fact that a female is laying eggs indicates that she is receptive (Fincke, 1997). In some instances if females have already copulated and hold enough sperms in their receptacles must avoid unnecessary copulation while ovipositing (Trivers, 1972); (Parker, 1979); (Thronhill and Alcock, 1983). If females that arrive at oviposition sites, often carry some sperm before they mate again, it is commonly assumed that females are not sperm limited (Waage, 1984a). Males are expensing their fitness in forming territory in anticipating that he may get chance for mating with females that enter there. He is in edgy environment to find mate. If female enters there is unreceptive she gives rejection signals to male. Males also heed a female's rejection signal after she has completed laying on a given day, so he remains a little chance that a his sperm would fertilize eggs in her later clutches. If the coercion with such females are taken in tandem at the water, they signal and/or actively resist males and are released in a matter of minutes (Kaiser, 1985); (Fincke, 1986a); (Forbes & Teather, 1994). Male may be aware with the receptiveness of female and generally avoids her for purpose of

mating with unreceptive because he need to spare his fitness. But if he was astir he can potter and grabs her. When females ultimately control the outcome of the interaction, male cooperation should minimize time and energetic losses to both sexes (Gorb, 1992). However, although male harassment directed towards ovipositing tandem pairs is costly to females and may even kill them (Robertson, 1985); (Hadrys et al., 1993); (Fincke, 1994a). Fitness costs of harassment to solo females is not well documented (Forbes, 1991). The selection of healthy habitat for eggs may be one of the most important arbitrate of a female, because the decision can affect her own survival as well as the success of her eggs (Rausher, 1979); (Lima & Dill, 1990); (Magnhagen, 1991). In the present study we reported mating behaviour of female dragonfly *Orthetrum sabina* that how she agreed or not for mating with a male in different situations.

MATERIAL AND METHODS

Study area: It was the area selected where whole study regarding reproductive behavioral aspects of selected dragonflies *Orthetrum sabina* was carried out during research period. The area was selected as the rivers, brooks, ponds from agricultural fields around Amravati city. Area was divided in two zones north and south side of National Highway number 6. The events of these behavior found were recorded at the bank of Kathora river near Rahatgaon, Amravati, Maharashtra. It is area of agricultural fields near ring road of Rahatgao village. Its geographic location on map was 20°58'38.4"N 77°46'33.8"E. Survey –As life cycle of dragonflies in both larval and adult stages is related to aquatic habitat, they at adult stage aggregate near water body mainly for their reproductive activities. With this purpose the wet lands were surveyed for the occurrence of the selected species in all seasons of the year.

Figure 1. Study area



Methodology: The selected different habitat for reproductive behavior in dragonflies were searched for *Orthetrum sabina* a species of Odonata from *libelulidae* family.

- Species identified in field with standard identification keys and pictorial hand book.
- The reproductive behavior of *Orthetrum sabina* for territorial behavior, mating , oviposition were studied. Observations were recorded in the form of photographs, audio recordings, videos and observed with binocular were taken to analyze the further data.

RESULTS AND DISCUSSION

Case I– in this case usual copulatory events of Orthetrum sabina were recorded. Pre mating event– Male-male interactions and territory guarding behavior: Male was seen to appear in the rendezvous from early morning, precisely from 7.40 am - 8.00 am. It changed accordance with season and climate. To make the asylum for feeding and mating male defended its territory with fervency. The males showed a virile territorial behavior. The territorial range was beyond which resident male did not respond to the intruder male. Here for *O. sabina* the average range of territory was found to be 3- 8 meters. Resident male made an expulsion of rival or intruder male by hovering and chasing the one who visited for invasions of territory. Intruder may be either conspecific (Plate No.1) or of heterospecific (Plate No.2) sometime. Resident male chased the intruder, kept watch on it by seating at different perches in the territory. Particular choice of perch was seen, as few perches were selected as favorite for supervision. Resident male tried to monopolize the area of his selection. While male- male interaction resident and intruder interestingly showed a forward, backward, downward and upward flying acrobatics in territorial area.

Male territory: Male was setup his territory at the river near a dense vegetation around. He was pacing his seating locations on the perch nearby

at three locations. He was seen to hover in and around his territory and defending it from rivals. He was seating on perch by dropping down his wings, my showed his alertness. He was setup his territory area circular about 6 meter in diameter. After one hour of hi pre reproductive activities he monopolize that area and waiting for receptive female. As he was become astir and ready for mating was seen by his activities. Grasping of Female : As soon as female entered territory of male, he suddenly face her with wing vibration the grasped female by using his abdominal appendices on her head behind her eyes, the grasping event was specifically marked with wings flapping sound like buzzing sound. The pair then flew towards perch in or around territory for matin Females Acceptance and copulatory wheel formation: If female was willing for mating

Plate 1. Territorial male making expulsion of conspecific rival male

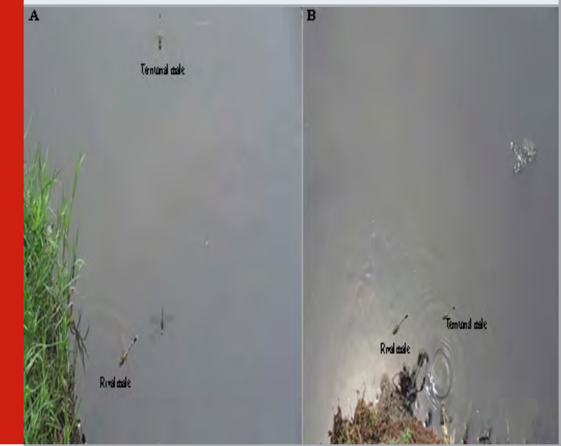


Plate 2. Territorial male making expulsion of heterospecific rival male.

then female curls her abdomen under her body to collect the sperm from the male's secondary genitalia that is at the situated on the sternites of segments 2-3 on his abdomen. Female locked her abdomen too forms a copulatory wheel 'tandem'. This event was complied within 4-5 second.

Sperm Transfer: Male rested on a perch with female in copulatory wheel (Plate No. 3) position, and transfers his sperms from secondary genitalia to female genitalia (sperm-storage organs) and fertilization apparatus which were located between the eighth and ninth abdominal segments. The transfer of sperm was takes place in different time duration depending upon the disturbance or need of sperms quantity by females. We observed in case of *O. sabina* the time of copulations lasts from 0.5 min -56 min. in our record.

Breakage of copulatory wheel: When requirement of sperm was over or due to any disturbances male and female get separated from each other and female moved towards water body for oviposition.

Plate 3. Male-female in tandem and male transfer sperm

Plate 4. In *O. sabina* mate guarding behavior

Post mating events.-Mate Guarding: In *O. sabina* it was found that male generally guard mated female while ovipositing. The non-contact guarding was found in *O. Sabina* male hovered over ovipositing female. It hovered over female in different directions showed his alertness and protected her from intruder males (Plate No. 4). Intruder was seen to be conspecific or heterospeccific male was. Male was so aggressive while female was ovipositing. Male's non-guarding decision also was observed in some cases.

Oviposition: As soon as mating was over female moved towards water body for oviposition. Female spontaneously stated egg laying in the water body. The oviposition site was mostly the territory of mated male, or by chance related to disturbance it might be site beside to territory. We observed exophytic oviposition in this species. We found two way of exophytic oviposition in *O. sabina* i.e. i) laid eggs by washing its abdomen on water surface. ii) Laid eggs on submerged vegetation (Plate No. 5). Female flicked her ovipositor on surface where she desired to lay her eggs. She continuously laid her eggs in batches. She bulwarked herself from faze of other males and continuously She was frequently changing her locations of egg lying. She laid 41 to 150 clutches of eggs, its duration was from 1min – 3.5 min was noted. No underwater oviposition was observed.

Case II- In this case female of *Orthetrum sabina* refuses mating and acted as death feigning was recorded.

Plate 5. Female of *O. sabina* ovipositing in different way

Mating: Suddenly a conspecific female enters the territory. Male was eagerly waiting for this opportunity. He quickly fly in front of her, hover above grabbed her wings by his legs and seize her with his anal appendages behind her head. He then turns towards the perch in the territory. All this events get completed in just few seconds. He was seen to waiting for females turn to help in making tandem wheel by adjusting her abdomen near his the copulatory apparatus at the second and third segment. But his expectations get failed, as female refuse to do so.

Mating refusal: In general after grasping female by her male she helps him for flying at perch and to make copulatory wheel the 'tandem' for copulation. On saturday, January 06, 2018, 1:25PM it was observed that the female he grabbed was evasive to make tandem and appeared to be resisting the males grasp by her wing vibration. She got hanged to males abdomen inactive for 7 min in dead position. Although she get injured her wings by vibrating she tries to escape and get freed from the grasp of importunate male without copulation. One failure of copulation added to account of that territorial male and female get freed from his grip and ran away.

DISCUSSION

We also investigated that species studied were found territorial in behavior and defending it from intruders and rivals of conspecific and heterospecific males. Numerous similar studies (Lutz and Pittman, 1970; Baird and May, 2003; Worthen and Patrick, 2004).were conducted on communities of dragonflies that have found more intraspecific contests. We found in our

Plate 6. Male waiting in his territory for female

Plate 7. Female hanged out in dead position

Plate 8. Female vibrates her wings to escape

investigation that if female was willing for mating then female curls her abdomen under her body to collect the sperm from the male's secondary genitalia that is at the situated on the sternites of segments 2-3 on his abdomen. Female locked her abdomen too forms a copulatory wheel 'tandem'. This event was complied within 4-5 second. Similar willingness of female and coercion of male was also observed in a study by Waage (1984) he reported that in Odonates, the males have to persuade the female to mate in tandem and the female should be willing to engage her genitalia with the males. We observed female *Orthetrum sabina* got hanged to males abdomen inactive for 7 min in dead position. Although she get injured her wings by vibrating she tries to escape and get freed from the grasp of importunate male without copulation showing her refusal towards mating. Similarly Waage, (1979, 1984), reported that unreceptive females react to courting males through the refusal display by spreading all four wings and raising the abdomen.

CONCLUSION

We conclude that in first studied case both male and female was matured and had willingness for mating so all proper reproductive events were followed by them. But in second case the female may be immature and not ready for mating, the male was not of her choice because he has poor territorial site, may be the eggs deposition by her would have already been accomplished for the day. Or female may have already carried sperm so she rejected that territory owner. She could still lay her eggs when the territorial male is absent, or can use another site.

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Comparison of MDAMB-231 Cells Cultured Under Different Conditions on 2D and 3D Silk Scaffolds

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ABSTRACT

In the last few years, bioengineered tumor models for breast cancer has revitalized academia and industries with better tools to assess dynamic features such as cancer progression, invasion and metastasis. Previously, breast cancer biology has been studied predominantly by means of two-dimensional (2D) cell cultures, which were deficient in mimicking the tumor microenvironment (TME). To alleviate such limitations, three-dimensional (3D) tumor models have been proposed, which are cost-effective and reliably reproduce the complexity of the breast-cancer TME. We have developed two types of silk scaffolds, HFIP-based sponges and lyophilized, to mimic the TME. Silk fibroin, for fabricating the scaffold, was extracted from Bombyx since they are known to be biocompatible, tough, elastic, and biodegradable. We observed, that MDAMB-231 cells cultured in these scaffolds have altered proliferation, stemness, hypoxia and propensity to transition from an epithelial to a mesenchymal phenotype. This 3D breast cancer model can be a cost-effective alternative and could be used to study the molecular mechanisms and impact of drugs.

KEY WORDS: SILK FIBROIN SCAFFOLD, 3D CELL CULTURE, TUMOR MICROENVIRONMENT ANALYSIS, BREAST CANCER CELL MODELS.

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INTRODUCTION

The exponential rise in cancer and allied issues globally has revitalized academia and industries to explore and achieve efficient tools and methodologies for early and effective diagnosis. Amongst the major efforts, in-vitro tumor models as been appreciated as a potential tool for understanding cancer biology. It contributes towards the screening of efficient anti-cancer agents Chang (2008). In-depth exploration shows that majority of the in-vitro researches use monolayer culture (2D) for cancer cell development. Such conventional paradigms are also known as Cancer Cell Monolayer Cultures (CCMC). However, such approaches exhibited significantly high limitations due to lack of tumor-specific microenvironments Chang (2008), Vinci (2012), Erler (2009), Polonia-Alcala (2018). To the drawbacks of 2D scientific community explores in vitro models to mimic the microenvironment augment their efficacy in preclinical trials. To achieve such objectives, the use of Three-Dimensional (3D) structure for optimal spatial growth of cells would be of paramount significance .

The key differences in cell phenotypes and molecular signature when cells are cultured on 2D and 3D structures has led to the proliferation of 3D cell-based platforms in biosciences. 3D cell culture platforms typically vary as per material's characteristics, processing mechanisms, and their structures. For example, the key 3D cell culture platforms are reconstituted ECM, synthetic hydrogels, porous polymer scaffolds, and nano-topography. Previous studies, Ravi (2015), Lee (2007), Edmondson (2014), Wang (2018), Bai (2019) have shown that the numerous 3D cell mechanisms, including spheroid, hydrogel or scaffold-based cultures, can enable environmental cues in the same way as observed in physiological or pathological tissue. Though, 3D cell characteristics can be easily reproduced across the different solutions of the 3D cell-based platforms, it is inevitable to define 3D cell-based platforms as complementary or integrated tool to perform cancer microenvironment analysis. Varied illustrations of the accessible 3D cell culture platforms can be visualized as a methodology with the potential to inculcate in-vitro conditions self-assembly, perfusion, and co-culture with other studies have suggested that tumors should be viewed as a well-structured pathological organ Egeblad (2010) consisting different types of cells like fibroblasts, endothelial cells, immune cells or adipocytes Hanahan (2012) rather than a mass of unrestrained proliferating cells. This alternate viewpoint broadens the horizon for in vitro models by co-culturing of cells with varied origins. Early research had revealed that co-culture methods show significant changes in different biological mechanisms like epithelial to mesenchymal transition, metastasis, neo-angiogenesis, fibroblast's transformation into Cancer- Associated Fibroblasts (CAFs) Kim (2015), Angelucci (2012), Sethi (2015), Esendagli (2014), Sung (2013). Similarly, co-culture mechanisms have exhibited the transformation of macrophages into Tumor-Associated Macrophages (TAMs) Kim (2015), Angelucci (2012), Sethi (2015), Esendagli (2014), Sung (2013). However, there exists large scope for understanding the pathology of tumor microenvironment, which can be vital for the development of new and effective cancer therapies.

Considering all this, in our work, the emphasis is made on the development of a 3D breast cancer model by employing a natural silk scaffold. Silk fibroin fibres have been employed in medicine since a long time, especially as surgical sutures. However, its significance as a biomaterial has increased many-folds in the last few years due to the development of silk-based 3D scaffolds for cell cultureKearns (2008), Altman (2003), Jastrzebska (2015). Considering its robustness especially in terms of biocompatibility, biodegradability and the self-assembly, it has been applied in numerous bio-engineering purposes including the development of cartilage and bone tissues Sundelacruz (2009), Yodmuang (2015). In the last few years, tumor models for hepatocarcinoma Kundu (2013), and mammary adenocarcinoma Talukdar (2011), osteosarcoma Tan (2011) have been developed using silk scaffolds. Few researches and allied only models which have used three-dimensionality of the tissue as well as heterotypic interactions between cells Chiew (2017), Onion (2016), Amann (2017), Valdez

(2017). In this study, we have cultured cells with three different conditions each on 2D, plain silk layers and 3D silk scaffolds as mentioned below.

1. MDAMB-231 cells as control,
2. Conditioned Media- MDAMB-231 cells are cultured in media taken from flasks containing 3T3 cells. This media is called conditioned media as it contains the proteins and other factors secreted by 3T3 cells, which could influence MDAMB-231 cell growth like in co-culture conditions,
3. 3T3-MDAMB-231- In this condition 3T3 cells are cultured/grown on the scaffolds for 24 hr, after 24 hr 3T3 cells are removed using 2mM EDTA (EDTA helps in cell detachment from the surface but the ECM secreted by the cells stay intact on the cultured surface). MDAMB-231 cells are then cultured on these ECM-coated scaffolds.

In order to facilitate 3D architecture, we used natural silk which was extracted from the cocoons of Bombyx mori. Standard protocols were followed for extracting silk protein Fibroin from Bombyx mori cocoons and making scaffolds out of them. We developed two types of scaffolds; HFIP-based sponges and lyophilized scaffolds. In our work, we enhanced scaffold production mechanism along with cell seeding, long-term 3D cell culture and cell detachment mechanism. We have characterised our model by measuring the swelling and by using microscopy, cell proliferation assay and gene expression analysis. In this study, the consideration of the genetic modification after culturing cells under different conditions, gave efficient cell production along with better cells separation. Such labeling helped making optimal assessment of their reciprocal interactions by means of gene expression (patterns) analysis.

MATERIAL AND METHODS

We have developed two types of silk scaffold. HFIP-based sponges and lyophilized scaffolds which were obtained by extracting silk fibroin from silkworm Bombyx mori. Silk fibroin is biocompatible, tough, elastic, and exhibit better mechanical properties and biodegradability with tunable degradation rates. Unlike classical 2D culturing, we employed 3D culturing. Breast cancer cells were seeded on this naturally-derived biomaterial matrix for mimicking of the TME while assuring sufficient crosstalk amongst cancer cells and stroma. In this study, the morphological and proliferation properties of breast cancer cells grown on these scaffolds were evaluated. The detailed discussion of the materials and methods used in this study is given in the sub-sequent sections.

Silk-Scaffold Development: We first extracted silk-protein fibroin from Bombyx mori cocoons. To extract silk-protein fibroin from Bombyx mori cocoons, we followed the standard protocols proposed in Danielle (2011). We first degummed worm-free, clean cocoons, with predefined weight, by boiling them in 0.02M Na2CO3solution for 40 minutes.Once degummed, the fiber mesh obtained was washed three times for 20 minutes individually and then air-dried overnight under aseptic conditions. The dried silk fibers, we weighed and dissolved using 12% w/v silk/9.3M Lithidum bromide (LiBr) and maintained at 700C for four hours. To perform further dialysis the dissolved silk solution was added with LiBr into a dialyze membrane which was prepared before pouring the silk Libr mixture.The dialysis membrane was prepared as the standard protocol proposed by Rosenberg (1996). In this method, the required length of dialysis membrane was cut and boiled in 2%Na2CO3 +1mM EDTA for 10 minutes which was later washed thoroughly. Silk with LiBr mixture was poured to the dialysis membrane and was processed for 48 hours against Millipore water. Noticeably, during the dialysis process, the water being used was changed at a defined interval (1, 3, 6, 12 and subsequently every 12 h). After dialysis for 48 h, the solution inside the dialysis membrane was transferred into a falcon, which was centrifuged twice at 9000 rpm at 40C for 20 mins.

The clear supernatant was collected and stored at 4°C in fresh falcons while the debris was discarded. Before making scaffolds, we calculated the percentage as well as weight of silk in the

sample. In our experiment, the percentage of silk was calculated by adding 0.5 ml of the solution on aluminum foil, which was kept for drying at the temperature of 600C.Before adding silk solution with aluminum foil, it (i.e., aluminum foil) was weighted and recorded. Thus, obtaining dried silk with foil we weighted it and recorded. To calculate the weight of silk we used following equation.

$$Weight\ of\ silk(W_s) \quad (1)$$

= (Weight of dried silk with foil- Weight of foil)

Thus, obtaining the weight of silk, we calculated the percentage of silk using (2).

$$Silk\ (\%) = \frac{W_s}{0.5\ ml} \times 100 \quad (2)$$

In (2), 0.5 ml signifies the volume of silk solution considered for analysis.

Making of SFs: As already stated, in this study two distinct types of scaffolds were developed; HFIP based sponges and lyophilized scaffolds. To prepare lyophilized scaffolds 1ml of 4% silk fibroin (per well) was added in a 24 well plate and lyophilized for 24 hours. On contrary, to prepare HFIP based sponges (i.e., scaffolds), 7% w/v lyophilized silk/HFIP solution was prepared where silk fibroin was dissolved completely in the HFIP solution. During silk dissolving phase, NaCl was sieved for uniform sized 250-425 µm. 3.4 gm of NaCl was spread in 35mm dish. This process was repeated for as many scaffolds as required. To obtain a uniform layer the 35 mm dish with NaCl was tapped gently, followed by adding 1 ml of silk/HFIP solution per well using a syringe in the fume hood. Subsequently, dish was wrapped with parafilm and left in the chemical hood for 1-2 days for the silk/HFIP solution to penetrate through the salt.

Once the salt appeared wet i.e., the silk solution reached the bottom of the dish. The dish was left open in chemical hood for HFIP to evaporate for a day, which was then followed by adding 1ml of methanol to each container and sealed with parafilm and left for a day. Excess of methanol was removed from the sample. The containers containing samples were transferred to a two liter beaker with Millipore water and left for salt to dissolve. The water was changed 2-3 times per day and was continued for 3 days for salt leaching. Once the salt dissolved, the scaffolds were separated from the dishes and were collected in a falcon and stored in Millipore water at 40C until used. To prepare plain silk sheets (as control), 1ml of silk/HFIP solution was poured in 35 mm dish was left open in chemical hood for HFIP to evaporate for a day, which was then followed by adding 1ml of methanol to each container. The prepared sample was left for a day. Excess of ethanol was removed from the silk sheets and was dried.

Characterization of the developed Scaffolds: Once the scaffolds were ready for further assessment, we performed in depth characterization in terms of swelling property, water-uptake property, mechanical characterization, Scanning Electron Microscopy (SEM) analysis etc. A brief of the characterization methods applied in this study to assess efficacy of the developed 3D breast cancer model using Silk Fibroin Scaffold.

Swelling property of the scaffolds: To examine swelling property of the developed scaffolds, they were hydrated for 24 hr in ionized water and Phosphate buffer saline (PBF) separately and each scaffold was incubated at room temperature and 37°C. After 24 hr, the scaffolds were assessed for its swelling percentile, which was obtained using following equation (3).

$$Swelling\ (\%) = \frac{M_{Wet}}{\left(M/M_{Wet}\right)} \times 100 \quad (3)$$

In above equation (3), the variable M_Wetsignifies the weight of scaffold after 24 hours of soaking in water or PBS, while M refers the dry weight before soaking.

Water-uptake property of the scaffolds: Quantification of water uptake of the scaffolds were obtained by soaking scaffolds in ionized water and PBS seperately for 24 hours at room temperature, followed by drying at the 50°C. To calculate water-uptake property, we used the following equation (4):

$$Water - Uptake\,(\%) = \left(W_S - \frac{W_d}{W_S}\right) \times 100 \quad (4)$$

In (4), the variables W_S and W_d signifies the weights of the wet scaffolds and the dried scaffolds, respectively.

Mechanical Properties of the Scaffolds: To perform mechanical characterization of the developed SFs, we used Dynamic Mechanical Analysis (DMA) method. Typically, DMA is applied to assess elastic properties (bending, elasticity, tensile strength). DMA analysis was obtained for both the scaffolds i.e., HFIP based sponges as well as 4% lyophilized scaffold.

Cell culture: In this process, MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium—high glucose (DMEM, Sigma–Aldrich) which was supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma-Aldrich, North American origin) and antibiotics penicillin streptomycin powder (Hi Media) 0.4 µg /ml final concentration. The cells were cultured at 37°C, where the relative humidity was maintained at 95% with 5% CO_2. In our experiment, we used cells from an 80% confluent dish. Silk scaffolds and plain sheets where coated with collagen type IV, followed with PBS washes, the scaffolds where incubated in culture media overnight. Cells were seeded at the rate of 1, 00,000 cells per scaffold/ plain silk sheet. In this experiment, cells were cultured under three distinct conditions on both scaffolds as well as plain silk sheets (control) as explained in the introduction section.

Biochemical or Molecular Assay: Once obtaining the distinct cell culture samples, we perform molecular assays where each sample was processed or examine for DNA quantification and RT-PCR. A snippet of the DNA quantification and quantitative real-time polymerase reaction is given in the sub-sequent sections.

DNA Quantification

In this study, the proliferation of the cells was assessed under control and treated conditions, which was evaluated at day 1, 3 and 5 by measuring the DNA content using the Pico-green dsDNA quantification kit (Invitrogen). To perform DNA quantification, we used a recent protocol proposed in Kumar (2016). Summarily, the cells were lysed using lysis buffer (0.02% SDS with Proteinase K 0.2 mg/ml). The lysate was mixed with the Picogreen dye to determine DNA content by measuring the fluorescence intensity in a well-plate reader with 485 nm excitation and 528 nm emissions.

Quantitative real time polymerase reaction: To assess quantitative real-time polymerase reaction, cells were cultured on silk scaffolds and control plain silk for two different time points 7 days and 14 days. For molecular gene expression studies RNA was isolated using the RNase Mini Kit (Quigen) as per the manufacturer's instruction. In

Table IA Swelling Property (in Percentile)

Temperature	Hydration	
	Water	PBS
Room Temperature	95.2	95.0
37°C	94.7	95.4

Table IB Water uptake property (in Percentile)

Temperature	Water uptake (%)	
	Water	PBS
50°C	94.9	92.9

this study, we used a total of 2 µg of RNA for cDNA synthesis using a high-capacity cDNA reverse transcription kit (Applied Bio-systems) as per the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was carried out using a Power up SYBR Green master mix (Thermo Scientific) with 10ng of the cDNA as the template. Further, we normalized the gene expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the fold change was calculated by means of 2-ΔΔct. To study gene expressions, we used EMT markers Epithelial-cadherin (E-cad), Neural-cadherin (N-cad), Vimentin, Snail, Slug, Twist, Paxillin, along with Matrix metalloproteinase-2 (MMP2), Sox2 and HF1 primers.

RESULTS AND DISCUSSION

We have characterized the mechanical properties of the developed silk scaffold and characterized the biochemical properties of the cells grown in them through molecular assays such as DNA quantification and Quantitative real time polymerase chain reaction. We have used plain sheet of silk as control for our biological assays onHFIP + silk scaffolds. We further discuss the results obtained and our inferences. Since porosity is a biologically-relevant mechanical property, we have characterized it by measuring the swelling (Table IA) and water-uptake (Table IB) of our scaffold. Swelling was measured at room temperature and at 37°C for water and PBS.

The swelling at room temperature was higher when hydrated with water in comparison to hydration with PBS. Interestingly, at 37°C, swelling was higher when hydrated with PBS We estimated water uptake at 50°C using equation (4) (Table IB). We observed that the water uptake under water is higher than with PBS These results show that these porous scaffolds are suitable for 3D tissue culture. Such scaffolds can be used to understand various properties of the cell such as adhesion, polarity, and morphology. The stiffness of silk/HFIP sponges is higher (5.8 kPa) in comparison to 4% lyophilized scaffold (2.8 kPa). Moreover, the stiffness of thesilk scaffold is closer to the stiffness of the breast tissue in vivo when compared to the stiffness of standard tissue culture dishes, which is in the order of gigapascals Paszek (2005),.

This is relevant because mechanical forces and stiffness of the cell microenvironment is known to alter gene expression patternsand cell signaling. In addition, the rigidity of the matrix can also affect stem cell separation, the migration of cells through cell membrane receptors, and activation of actin cytoskeleton Engler (2006), Mendoza (2010), Menezes (2008), Buxboim (2010), Erler

Figure 1. Scanning electron microscope images of silk scaffolds prepared using the HFIP at 100 and 50 micros.

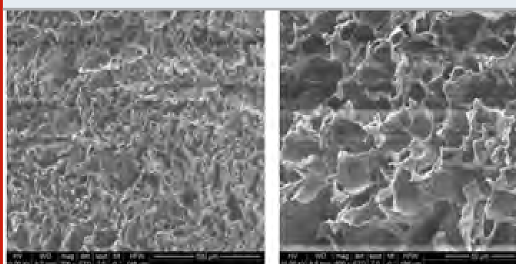
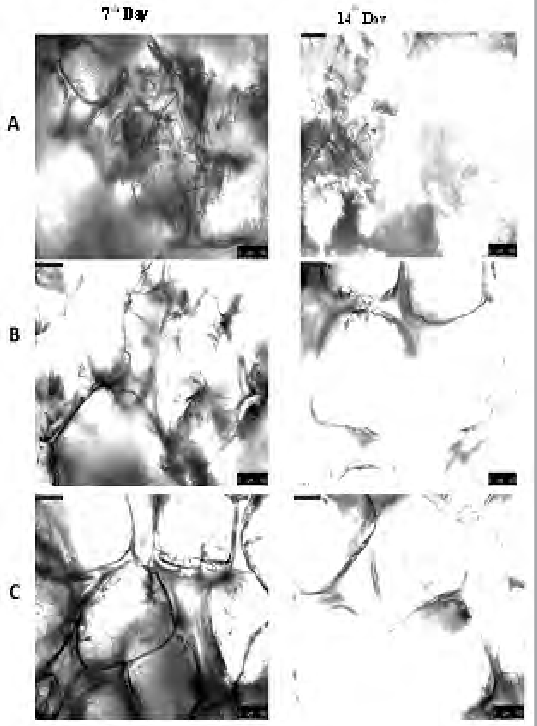


Figure 2. Scanning electron microscope images of cells cultured on silk scaffolds with (A) MDAMB, (B) Con-MDAMB, (C) 3T3+MDAMB at 100 micros.



(2009), Kim (2009). the porous nature of the scaffold enables circulation of oxygen and nutrients that could eventually help in elimination of cell detachment and its debris. On contrast, such features are not visible in the 2D monolayer culture that typically exhibits cellular uniformity (in terms of shape and size) along with contact inhibition at confluence. Hence, we have chosen HFIP-silk scaffolds for further experiments. Cells cultured on the 3D scaffold typically have more space to proliferate and hence take more time to reach confluence. Cells form clusters in between the single layers of cells towards the scaffold's surface which eventually gives rise to the multilayer clumps. Lack of detoxification results in growth arrest which then later develops into necrosis in the central regions. This mimics breast tumor in vivo and is therefore a more realistic breast cancer model. Figures 1 and 2 show the SEM images of the scaffold and cells cultured in them. Cells were cultured for a period of 7 and 14 days (Fig. 2).

We have measured the DNA concentration of MDAMB cells cultured on control and silk scaffolds and under three conditions, normal, conditioned media, and decellularized ECM matrix for a period of 3, 6 and 9 days. (Fig. 3) In the control at normal conditions, the proliferation increases from day3 to day 6 and then it decreases. In the case of MDAMB cells grown under conditioned media, the cell proliferation is consistently decreasing from day 3 to day 9 for both control and silk scaffolds. This could be due to the secreted factors present in the conditioning media. In contrast, the cells cultured on control and silk scaffolds containing on decellularized ECM matrices are proliferating. This shows the importance of cell-ECM interaction in tumor formation. The following results exhibit the quantitative real-time polymerase reaction assessment of the silk scaffolds. Fig. 4 a,b,c,d exhibit the molecular change in cell cultured on plain and scaffolds on 7th and 14th day. We have characterized the fold-changes of different EMT markers; Epithelial-cadherin (E-cad), Neural-cadherin (N-cad), Vimentin, Snail, Slug, Twist, Paxillin, along with Matrix metalloproteinase-2 (MMP2), Sox2 and HF1 primers.

Results (Fig. 4) reveal that cells cultured on plain scaffold showed both epithelial and mesenchyme characters, on 7th day increase in E-cad and Paxillin levels contributing to the epithelial character but on the 14th day change in levels of slug, snail, twist, MMP2 along with N-cad and Vimentin contributing to mesenchyme character, with increase in stemness and hyoxia condition contributes to processes involved in metastasis like chromosomal instability, invadopodia, angiogenesis etc. Cells on scaffolds with time showed mesenchyme characteristic. Gene expression levels of Slug, Snail and twist contributes to increase in cell stemness (SOX2) and metastasis.

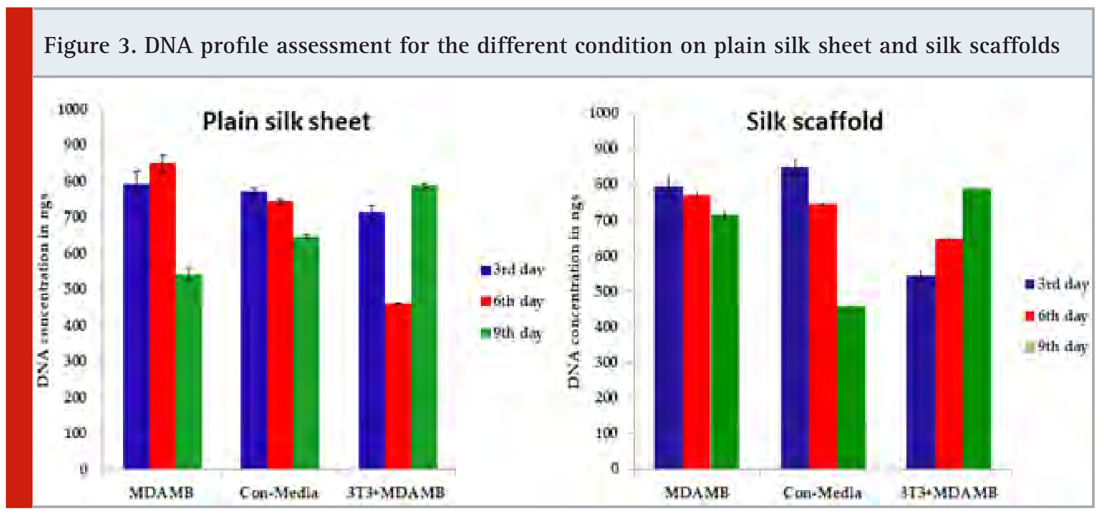
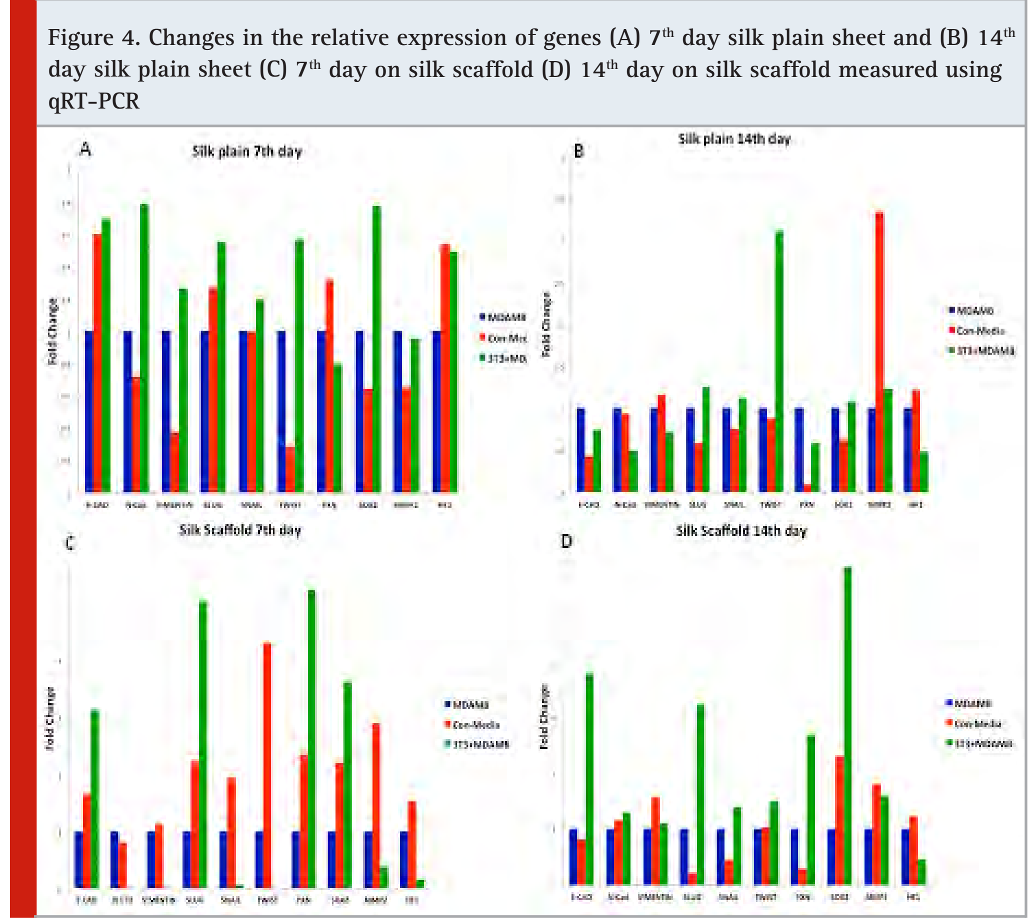


Figure 3. DNA profile assessment for the different condition on plain silk sheet and silk scaffolds

DISCUSSION

The key limitations of the major at hand approaches or cell models are its inability to examine the efficacy of anti-cancer agents and varied cell-interactions occurring in the TME. Such limitations revitalize researchers to develop more efficient and advanced in vitro tumor models. In our work, a novel breast cancer model (3D in vitro culture model) was developed for mimicking the architecture and the stiffness of the breast tumor. This approach enabled spatial cell-development on a porous, scaffolding structure that mimics the in vivo tissue-like microenvironment for the cells. The concurrent use of different culturing conditions for cells characterizes robustness of the proposed model to form a "tumor organ". Such culture mechanism enabled the in-depth assessment of the direct as well as reciprocal interactions amongst the cancer cells. The bio-material used in this cancer cell model was natural silk fibroin, which is cost-efficient, accessible, non-toxic that after removal of sericin layer exhibits no activating adaptive immune response. Initially, we emphasized on selecting the best scaffolding technique for breast cancer tumor cell's culture. In our study, we exhibited that silk fibroin scaffolds with the pore size ranging 250-500 µm can be significant towards optimal cell proliferation and associated infiltration. Here, two commercially available cell lines were used for 3D culture. Considering the significance of the TME in cancer biology, different in vitro culture models have been proposed Belli (2018), Kolenda (2018). However; not much significant efforts are made towards assessing direct as well as reciprocal cell interactions analysis that often takes place in between stromal and tumor cells. There are some cancer models which employ indirect co-culture, by applying cell-growth within distinct compartments or by using conditioned media (CM) from the specific kind of cells. Such approaches

Figure 4. Changes in the relative expression of genes (A) 7th day silk plain sheet and (B) 14th day silk plain sheet (C) 7th day on silk scaffold (D) 14th day on silk scaffold measured using qRT-PCR



enable merely understanding the impacts of paracrine signaling but avoid exhibiting direct cell-to-cell interactions. In this study we developed a system that enables assessing direct cell-to-cell interactions. The scaffolds allowed cell attached, proliferating and ECM secreation, affirming the fact that silk fibroin can be a potential and better biomaterial to support cell culture. This study revealed perceptible differences in morphology (in cells cultured in 3D culture). It is found that in the culture cell model, it becomes intricate to distinguish singular cells by SEM images due to cell-embedding into the thick layer of extracellular matrix generated by cells. In addition, this study revealed different growth kinetic and morphological changes in 3D culture model. The gene expression patterns of the cells were examined. Cells cultured on 2D silk sheets, were characterized to be more epithelial phenotype when compared to cells cultured on 3D scaffold.

Factually, such phenotypic inferences depend predominantly on the morphology of cell. In the considered breast cancer cells, breaking of β-catenin often cause significantly rise in cell mobility along with mesenchymal, vimentin expression that refers an EMT Yamori (1997). The cell-transition into a 3D environment signifies their higher mesenchyme phenotype nature. Noticeably such features were observed more in those genes which are conscientious towards extracellular matrix production and remodeling. Thus, this study revealed that the 3D culture enables cells the environmental cues required to maintain their physiology Yamori (1997).

CONCLUSION

We have developed and characterized a silk-protein-based scaffold for mimicking the tissue microenvironment and stiffness in vitro. Breast cancer cells (MDAMB-231) were cultured with ECM secreted by fibroblasts (3T3) on these scaffolds. Two different scaffolds, namely, HFIP-based sponges and lyophilized scaffolds were developed. The geometry of the scaffolds was characterized using SEM; physical properties such as swelling, and water-uptake were measured. We characterized the cells cultured on these scaffolds by measuring their morphology, proliferation and gene expression levels of a panel of cancer-associated genes. Our preliminary results show that cells in the scaffold show enhanced expression of genes associated with invasion, migration, ECM-remodeling and metabolism. It is pertinent to note that even though MDAMB-231 cells are metastatic, they gradually lose their mesenchymal phenotype when grown in 2D culture dishes. In contrast, when grown on our scaffolds, they exhibit their true metastatic potential. Moreover, our scaffolds are flexible, cost effective, biocompactable and easy-to-handle. Hence, our system could be an attractive in vitro model for studying breast cancer metastasis in vitro, testing the efficacy and molecular mechanisms of the ani-cancer drugs.

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Impact of 17α- Hydroxy-Progesterone and Eyestalk Ablation on Ovarian Maturation in Relation to Protein Changes in the Ovary and Hepatopancreas in Freshwater Crab, *Barytelphusa cunicularis*

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ABSTRACT

The present work was planned to determine the effect of 17α-hydroxy progesterone hormone and eyestalk ablation on ovarian maturation in relation to protein changes in the ovary and hepatopancreas of freshwater crab, *Barytelphusa cunicularis*. The studies showed that, administration of 17α-hydroxy progesterone hormone indicated considerable changes in protein content during ovarian maturation over base control and experimental control crabs. In the present examination eyestalk ablation showed increase in ovarian maturation over base control, experimental control and hormone treated crabs by elevating the mobilization of biochemical constituent(s) from hepatopancreas to the ovary. Moreover, eyestalk ablation method found more significant than hormone injected which may be used for enhancement of crustacean culture.

KEY WORDS: *BARYTELPHUSA CUNICULARIS*, OVARIAN MATURATION, PROTEIN, 17α-HYDROXY PROGESTERONE

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INTRODUCTION

Population explosion, pollution, climate change, unplanned management and other factors increases pressure on the agricultural productivity which reduces access to food quantity and quality. So to fulfill the need of the people, aquaculture now a day's is a good and reliable source particularly crustaceans; which is an important source of aquatic food protein, ornamental and trade which are extremely important for any developing countries. It provides both economic development and empowerment in terms of contribution to GDP, consumption, employment, catch value and exports. The crustacean sector generates high value export products which enables producers to buy lower value products in the world market which is a positive contribution to food security in both producing and exporting countries (Bondad-Reantaso et al. 2012). Enhancement of aquaculture entirely depends upon reproduction which is the most fundamental and essential high energy demanding physiological process in living organisms to continue its race in which gonad maturation plays a vital role; whereas some species require external manipulation for it. The gonadal development can be stimulated or inhibited by affecting the neurosecretory cells by many pharmacological agents and eyestalk ablation (Subramoniam 2000; Lafont and Mathieu 2007; Coccia et al. 2010; Alejandra et al. 2011,Yang Lu et al 2018,Raghavan and Ayanath 2019).

Likewise, steroid hormones are biologically active in crustaceans found in the hepatopancreas, ovary and haemolymph which control vitellogenesis (Stevenson et al. 1979; Quackenbush 2001). Vitellogenesis is an important physiological process associated with female reproduction, because the primary source for the developing crustacean embryo is yolk protein and is a pivotal stage during crustacean reproduction. It is the synthesis of yolk proteins i.e. Vitellin (Vn) and Vitellogenin (Vg) which are the two main yolk proteins, that are important nutritive sources, which are necessary for the proper maturation and development of the oocytes (Tseng et al. 2001; Zapata et al. 2003). In decapod crustaceans, hepatopancreas acts as center for storage and / or synthesis of biochemical material which is transferred to sites of gametogenesis for the purpose of growth, maintenance and reproduction (Adiyodi and Adiyodi 1970). The mobilization and accumulation of protein, lipid and glycogen reserves in several tissues have been documented in several crustacean species (Khayat et al. 1994; Harrison 1997; Tseng et al. 2001; Thomas et al. 2005). In this regard the researchers have reported the role of some vertebrate-type steroids such as β- estradiol and progesterone in ovarian maturation. Reddy et al. (2006) demonstrated that 17α- hydroxyprogesterone hormone induced ovarian growth and ovarian VTG synthesis in the fresh water crab, *Oziotelphusa senex senex*.

Muhd-Farouk et al. (2014) studied effect of vertebrate steroid hormones on the ovarian maturation stages of orange mud crab, *Scylla olivacea* and found enhancement in the ovarian maturation. Sujathamma and Dayakar (2015) observed effect of estradiol and 17α-hydroxyprogesterone on ovarian development of fresh water paddy field crab, *Oziotelphusa senex senex* and found that both hormones were influencing the gonadal growth. Kale (2017) found significant ovarian maturation by mobilization of protein from hepatopancreas to ovary under the influence of α- estradiol hormone and eyestalk ablation in fresh water crab, *B. cunicularis*. Yang Lu et al. (2018) examined the effect of exogenous estrogen on the ovarian development and gene expression in the female swimming crab.

Portunus trituberculatus and found that hormone injection probably indirectly stimulate the ovarian development and vitellogenesis by mediating the secretion of hormones and gene expression in the endocrine organs. Raghavan and Ayanath (2019) investigated the effect of ecdysteroids on oogenesis in the freshwater crab, *Travancoriana schirnerae* and found positive results. The eyestalk is also one of the critical factors know n to control crustacean reproduction by a series of inhibitory neurosecretory factors which effectively targets the ovaries and hepatopancreas (Laufer et al. 1998; Aktas et al. 2003). Hussain et al. (2014) studied the effect of unilateral eyestalk ablation in fresh water

prawn, *Macrobrachium lamarrei* and noticed to induce gonadal development. Samyappan et al. (2015) studied impact of unilateral eyestalk ablation on lipid profiles in fresh water female crab, *Oziotelphusa senex senex* which showed a marked decrease in the hepatopancreas and a significant increase in ovarian tissue indicating ovarian maturation. Sarojini et al. (2016) studied impact of unilateral eyestalk ablation on protein content in freshwater crab, *Spiralothelphusa hydrodroma* and found significant increase in ovarian maturation. Recently, Rana (2018) revealed that eyestalk ablation is the alternative technique in induced breeding for rapid ripening of ovaries to enhance meat yield in aquaculture in freshwater crab, *Barytelphusa lugubris*. Available literature states that ovarian maturation is the process in which there is intense requirement of biochemical constituents like protein, lipid and glycogen which are transported from the storage organs under controlled hormone action. Present investigation was undertaken to confirm whether 17α-hydroxy progesterone hormone enhance the ovarian maturation in relation to synthesis and mobilization of protein from hepatopancreas to ovary and the results were compared with eyestalk ablation.

MATERIALS AND METHODS

Barytelphusa cunicularis were collected from Godavari River near Newasa and were acclimatized for a week in the laboratory. Healthy well-adapted female crabs of early-reproductive phase of approximately same weight and size of intermoult stage ranging between 35-40g body weights were selected for the experiments. Water in the troughs was changed daily and crabs fed by small pieces of earthworm and bivalve on alternate days. Other parameters like temperature, pH, salinity, photoperiod etc., were maintained constant during experiment. The crabs were divided into four groups, as base control, experimental control, 17α-hydroxy progesterone injected and eyestalk ablated group, each containing 10 crabs. The final concentration of the hormone preparation was 1µl = 1µg. The hormone was injected into the abdominal musculature of arthropodial membrane through 3rd walking legs, receiving a dose of 20µl /crab with the help of hypodermic syringe having a 27-guaze needle.

Preparation of 17α- hydroxy progesterone hormone injection: 10mg of 17α-hydroxy progesterone hormone was dissolved in 1ml of 1% ethanol and resulting solution diluted to 10ml by adding glass distilled water. The final concentration of the hormone preparation was 1µl = 1µg. From this preparation hormone was injected to crab receiving a dose of 20µl hormone/crab. The used hormone was obtained from Sigma Chem. (USA). The experiment was conducted for a period of 21 days; base control crabs were sacrificed on o-day of the experiment and remaining grouped crabs were sacrificed on 21st day of the experiment. Ovaries and hepatopancreas were dissected out for estimation of protein levels by Lowry et al. (1951). Protein levels were calculated on dry weight basis and expressed as % mg.

RESULTS AND DISCUSSION

Protein levels in the ovary were found to be 28.81 %, 31.72 % and 45.12 % for base control, experimental control and 17α-hydroxy progesterone injected respectively. Eyestalk ablated crabs showed 50.38 % protein level. While protein content in hepatopancreas were noted to be 48.12 %, 47.19 % and 36.47 % for base control, experimental control and 17α-hydroxy progesterone injected crabs respectively. Eyestalk ablated crabs showed 26.98 % of protein level. Increased protein level in eyestalk ablated group was prominent when compared to protein levels obtained in hormone injected. Biochemical studies are very important from the reproductive as well as the nutritive point of view in any edible crustacean animals particularly protein content. Protein plays very important role in the growth and maintenance of the animals and it is easily digestible too. In the present study (Table 1 and Fig. 1) the protein levels in the ovary were found to be 28.81%, 31.72% and 45.12% for base control, experimental control and 17α-hydroxy progesterone injected respectively. Whereas eyestalk ablated crabs ovary showed 50.38% protein level. While protein content in hepatopancreas were noted to be 48.12%, 47.19% and 36.47% for base control, experimental control and 17α-hydroxy progesterone injected crabs respectively. Eyestalk ablated crabs showed 26.98% of protein level. The present findings

indicated that hormone injections and eyestalk ablation showed enhancement in the ovarian maturation by synthesizing or and mobilization of the protein and other biochemical constituents from hepatopancreas to ovary over the base control and experimental control which seems reasonable that decreases were a consequence of accelerated transport to maturing ovaries in which proteins and lipids are accumulated and hepatopancreas may be the source for these constituents circulated through the haemolymph (Kulkarni et al. 1979; Teshima et al. 1988; Okumura and Aida 2001). Similarly, the role of steroid hormones in controlling the process of reproduction has been studied by (Yano 1987; Quackenbush 1989a; Subramoniam 2017).

In the same ray of light Tsukimura (2001) studied the effect of 17 α-estradiol, progesterone and 17 α-hydroxyprogsterone on ovarian maturation of crustaceans and found positive correlation to protein synthesis and its uptake for the maturation in ridgeback shrimp, *Sicyonia ingentis*. Zapata et al. (2003) observed ovarian growth in the crab, *Chasmagnathus granulata* induced by 17α-hydroxyprogesterone and Juvenile hormone III. Sujathamma and Dayakar (2015) in their studies examined the effect of estradiol and 17α-hydroxyprogesterone on ovarian development of fresh water paddy field crab, *Oziotelphusa senex senex* and found that both hormones promotes the gonadal growth and reproduction in female crab. Muhd-Farouk et al. (2016) noticed that the administration of 17α-OH pregnenolone as well as 17α-OH progesterone stimulated ovarian index, oocyte diameter and ovarian maturity in *S. olivacea* Kale (2017) studied the influence of β- estradiol hormone and eyestalk ablation on protein metabolism in fresh water crab, *Barytelphusa cunicularis* and found ovarian maturation due to mobilization of protein from hepatopancreas to ovary. Recently, Raghavan and Ayanath (2019) investigated effect of ecdysteroids on oogenesis in the freshwater crab, *Travancoriana schirnerae* study indicated that 20-OH ecdysone can stimulate ovarian growth and maturation in all phases of the oogenic cycle. Present studies also reveals that decrease in protein level in hepatopancreas and increased protein level of ovary in eyestalk ablated group was prominent over hormone injected crabs.

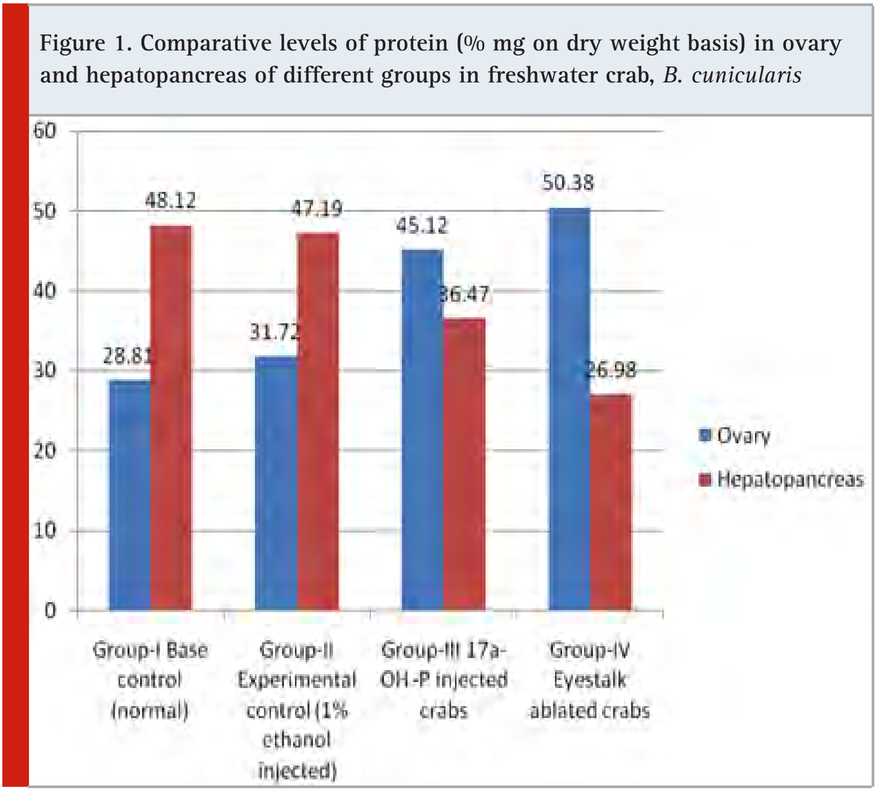
This may be due to the eyestalk ablation; because neuroendocrine system has long being known to play a significant role in gonadal maturation, and or moulting (Quackenbush 1986; Fingerman 1987). In the same way Arcos et al. (2003) reported similar results in shrimp, *Litopenaeus vannamei* suggesting decreases in total protein, cholesterol and triglycerides in haemolymph and ovarian maturation as an effect of eyestalk ablation. *Murugesan* et al. (2008) noticed eyestalk ablation influenced protein, lipid and carbohydrate content in ovarian maturation of crab, *Charybdis lucifera*.

Table 1. Shows levels of protein (% mg on dry weight basis) in ovary and hepatopancreas of different groups in freshwater crab, *B. cunicularis*.

Animal Category	No. of Crabs	Exp. Day	Tissue	Protein (%mg)
Group-I Base control (normal)	10	0-day	Ovary	28.81 ± 0.03
			Hepatopancreas	48.12 ± 0.31
Group-II Experimental control (1% ethanol injected)	10	21-day	Ovary	31.72 ± 0.34
			Hepatopancreas	47.19 ± 0.23
			Ovary	45.12 ± 1.07
Group-III 17α-OH -P injected crabs	10	21-day	Hepatopancreas	36.47 ± 0.82
			Ovary	50.38 ± 0.28
Group-IV Eyestalk ablated crabs	10	21-day	Hepatopancreas	26.98 ± 0.89

± S. D.: Mean Standard Deviation
17α-OH –P: 17 α- hydroxy progesterone hormone

Figure 1. Comparative levels of protein (% mg on dry weight basis) in ovary and hepatopancreas of different groups in freshwater crab, *B. cunicularis*



Varalakshmi and Reddy (2010) found significant changes in carbohydrate, protein and energy leading to ovarian maturation in *Macrobrachium lanchesteri* due to eyestalk ablation. Unilateral eyestalk ablation in freshwater female crabs, *Oziotelphusa senex senex* showed marked decrease in lipid classes in the hepatopancreas but significant increase in ovarian tissue which suggests ovarian maturation (Samyappan et al. 2015). Lee et al. (2017) and Guan et al.(2017) have also mentioned that eyestalk ablation is considered as the most effective method to facilitate molting and quick ovarian maturation in captive economic crustacean culture. Rana (2018) studied the effect of eyestalk ablation in freshwater crab, *Barytelphusa lugubris* and found that eyestalk ablation not only promoted the process of moulting but it also amplified the process of ovarian maturation. Similarly, Shabnam Banoo et al. (2018) also noticed increased gonadosomatic indices and gonad maturation of freshwater crab, *Paratelphusa hydrodromous* due to unilateral eyestalk ablation. Mhd Ikhwanuddin et al. (2019) found increase in gonadosomatic index and mean oocyte diameter which indicated ovarian maturation due to eyestalk ablation of blue swimming crab, *Portunus pelagicus*. But, Nagathinkal et al. (2017) studied molt and reproduction enhancement together with hemolymph ecdysteroid elevation under eyestalk ablation in the female fiddler crab, *Uca triangularis*. Their results indicate that throughout the annual cycle, both the somatic and the reproductive growth of *U . triangularis* are under the influence of inhibitory principles from the eyestalks. It is also revealing that mere deprival of the inhibitory principles does not culminate into successful vitellogenesis. Arguably, the inhibitory influence from the eyestalks could be a prerequisite for normal healthy maturation of the oocytes and spawning. In conclusion, from the available literature and results it seems that transfer of protein from hepatopancreas to ovary for its maturation was due to eyestalk ablation. It showed significant progress over experimental and hormone injected crabs which suggests that hormonal manipulation of crustacean reproduction is limited to eyestalk ablation for the induction of ovarian maturation till date and a new technological advances in such type

of hormonal manipulation and eyestalk ablation for understanding of crustacean endocrinology is essential for application of management programs for commercially important species in aquaculture.

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Allelopathic Effects of Alkaloid Contents of Hyoscyamus muticus and Withania somnifera on the Germination of Cichorium intybus seeds

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ABSTRACT

The aim of current research was to evaluate of the allopathic effect of aqueous extracts and alkaloid fraction of *Withania somnifera* and *Hyoscyamus muticus* against germination and growth of *Cichorium intybus*, in a laboratory. Alkaloid fraction and aqueous extract of aerial part of *W. somnifera* and *H. muticus* at different concentration, were applied to determine their effect on the seed germination and seedling growth of tested plant under laboratory condition. The germination and growth of *Cichorium intybus* were assessed using growth parameters. The results revealed that all the aqueous extracts markedly suppressed the germination and seedling growth of *Cichorium intybus*. *Withania extracts* showed remarkable effects on germination and the growth of *Cichorium intybus* in comparison to controls. The aqueous extract and alkaloid fraction of *W. somnifera* were more pronounced than *Hyoscyamus muticus* extracts in germination assay. All the aqueous extracts significantly suppressed shoot length, and root length, of *Cichorium intybus*. The inhibition of germination and suppression of growth parameters could be attributed to the presence of active phytochemicals compounds present in the extracts and alkaloid fraction which, subject to GC-MS analysis to study these compound, GC-MS revealed the presence of many active phytochemical compounds, of *W. somnifera* and *H. muticus* extracts (Ferulic acid, Methyl ferulate, Mandolic acid), Scopolamine and atropine are the major compounds in *H. muticus* with concentrations of 13.79% and 3.80% in alkaloid fraction, while in *W. somnifera*, Pterin-6-carboxylic acid are the major compounds with concentration of 2.5 %. The present study concludes that *W. somnifera* and *H.muticus* contain bio-herbicidal compounds in the plant extracts and alkaloid fractions which showed an inhibitory allelopathic effect on the development of wild chicory.

KEY WORDS: LLELOPATHY, *HYOSCYAMUS muticus* L. , *WATHENIA somnifera*, HARM SEEDS.

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INTRODUCTION

Allelopathy is formulated as any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of agricultural and biological systems. The allelopathic organism releases chemicals that inhibit the growth of a competing organism and thus indirectly prevents it from using common resources. For a long time, it has been recognized that several compounds that are present in the soil in replant situations, actively or passively alter the prevailing soil conditions, inducing a plant population, therefore, due to their allelopathic characteristics, these substances could somehow act as a pesticide (Awatief et al., 2013, Fang and Cheng 2015). *Withania somnifera* is a small shrub to 2 m high, the whole plant is covered with short, fine, silver-grey, branched hairs. The stems are brownish and prostrate to erect, sometimes leafless below.

The leaves are alternate simple, margins entire to slightly wavy, broadly ovate, obovate or oblong, 30–80 mm long and 20–50 mm broad, flowering time is mostly from October to June, while the fruiting time is mostly from October to July (Boulos, 1995). The medicinal importance of *W. somnifera* is due to presence of diverse secondary metabolites. The main chemical constituent of leave of W. somnifera are alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine), steroidal lactones (withanolides withaferins) and saponins (Atta-ur-Rahman 1996).Withania somnifera (L.) Dunal screened to investigate the biological activities i.e. antimicrobial, antioxidant, Anti-inflammatory, Anti-aging, Anti-carcinogenic, Cardioprotective, Hypothyroid, Pharmacological activities. The demonstration of broad spectrum of W. somnifera may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control (Kapoor 2001; Kalpana et al., 2014, Elsharkawy and Shiboop 2017).

Egyptian henbane (*Hyoscyamus muticus*) is one of the most important medicinal plant of the Solanaceae family. It contains valuable tropane alkaloids, hyoscyamin and traces atropine (Lavania, 2005). Tropane alkaloids, especially hyoscyamin and scopolamine, are widely used in medicine for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties. These alkaloids are synthesized in roots and then transported to aerial parts of the plant (Pal Bais et al., 2001). The genus *Hyoscyamus L.* belongs to the tribe Hyoscyameae Miers of Solanaceae family with 18 species all over the world (Yousaf, 2008) and 3 species in Egyp. *Hyoscyamus* species are rich sources of tropane alkaloids, mainly hyoscyamine and scopolamine, Tropane alkaloid have pharmacological and toxicological importance, and are spread in solanaceae family (Kartal et al., 2003). Fatemeh et al., (2012), determined scopolamine and hyoscyamine contents in five *Hyoscyamus* species including *H.niger* L., *H.reticulatus L., H.pusillus L., H. arachnoideus Pojark., and H. kurdicus* Bornm., collected from different geographical origins of North West of Iran by HPLC The range of genetic similarity was obtained between 91.07 and 99.89 within *Hyoscyamus* accessions based on scopolamine and hyoscyamine alkaloids composition.

Allelopathy is the science that studies processes in which secondary metabolites from plants and microorganisms are involved, affecting growth and development of biological systems (Singh et al., 2003) The use of secondary metabolites implicated in allelopathic interactions as sources for news agrochemical models could satisfy the requirements for crop protection and weeds management (Qiming et al., 2016). The aim of current research was to study positive or negative the allopathy of the application different concentration of aqueous extracts and alkaloid fraction of *Withania somnifera* and *Hyoscyamus muticus* against germination and growth of, *Cichorium intybus* in laboratory along with to also study the phytochemical compounds present in plant extract, where the alkaloid fraction was analyzed to understand the role of chemical composition of plants and their allophathic effects.

MATERIAL AND METHODS

Collection and Preparation of Samples: Plants for the study (*Withania somnifera* and *Hyoscyamus muticus*) were collected in June, 2016 from

the wild population in Alamain- Wadi El-Natrun Desert Road, Egypt. Seeds of plant weed (*Cichorium intybus*) were collected also collected from Farms of onion in near the plant and kept until germination process. The plant was identified in Desert Research center and the sample was deposited in the Herbarium of Desert Research center.

Laboratory Study: Water Extract preparation: 500 g of dried aerial part of each plants were boiled with 1000 ml distilled water for 2 hour, vigorously stirred and allowed to stand for 24 hours. These were then vigorously re-stirred and filtered into wash bottles until use, thereafter filtered using muslin cloth to obtain a stock solution of 0. 5 g /ml concentration. The stock solution was then adjusted accordingly to obtain four different levels concentrations i.e. 25%, 50%, 75% 100% . Distilled water was used as a control. 50 fifty Petri dishes double layered with Whatman No. 1 filter paper were taken and divided into two (2) sets, one set for Aqueous extracts and the other set for alkaloid extracts of roots. The same Experiment was done for each plant. Ten (10) seeds were sown in each Petri dish and moistened with 25 mls of an appropriate water extract of the weed. There were three (3) replications of each treatment (an aqueous and alkaloid extract). Twenty-five (25 mls) of distilled water was applied to the control. Filter paper linings of each Petri dish were moistened daily with an appropriate extract to prevent them drying up before final germination counts (10 days after sowing). The petri dishes were kept in a growth chamber at room temperature until the final germination count.Germination inhibition/stimulation Percentages of inhibition/stimulation effect on seed germination over control were calculated using the formula proposed by Singh and Chaudhary (2011).

Inhibition (-) or stimulation (+) = [(Germinated seeds in extracts - Germinated seed in control)/ Geminated seeds in control] x 100

Measurement of growth parameters: After the completion of seed germination, shoot length was measured using ruler with different days of developmental period. The root length and inhibition percentage of the root length for each seeds of weed was calculated. Germination data obtained were analyzed using analysis (ANOVA) test, the means of treatment were grouped on the basis of (LSD) at the 0.05 probability level. Preparation of Alkaloid fraction:100 gm of plant powder of each plant under investigation (W. somnifera and H.mutcus) was separately extracted by refluxing by 70% ethanol three time for 3 hours ,filtered of and concentrated by rotary evaporator then suspended in 200 ml water and separated in separating funnel, the filtered aqueous layer was fractionated by chloroform take chloroform fraction and evaporated to obtain (1.8 and 2.6 gm) from two plant ((W. somnifera and *H. mutcus*)) respectively, (Elsharkawy and Shiboop 2017). Preliminary Phytochemical screening: Preliminary phytochemical screening

Table 1: Effect of plant extract and alkaloid fraction on the germination *Cichorium intybus*

CONC %	*W. somnifera*		*H. mutcus*	
Germination % Plant Extract	Germination % alkaloid fr action	Germination % Water extract %	Germination % alkaloid fraction	
100	0%	0%	0%	0%
75	0%	0%	0 %	5%
50	0%	0%	5%	10%
25	10%	5%	10%	15%

Table 2: Phytochemical analysis of *Withania somnifera* and *Hyoscyamus muticus*

Class	*W. somnifera*	*H. muticus*
Alkaloids	++	+
Saponins	+++	++
Sterols and steroids	++	+++
Flavonoïd	_	+
Tanins	-	_
Anthocyanosids	-	_
Terpene	_	+

of water extracts for each plant for plant secondary metabolite (flavonoids, saponins, alkaloid, terpenes and tannins) was carried out using standard methods (Bao et al., 2005). GC-MS Analysis: Gas chromatography-mass spectroscopy (GC-MS) analysis GC-MS analysis of the plant extracts and fraction were carried out using a Clarus 500 Perkin – elmer (Auto system XL) gas chromatograph equipped and coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100% dimethyl poly siloxane), 30 m × 0.25 mm ID × 1 μm of capillary column. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization system operated in electron impact mode with ionization energy of 70 ev.

Table 3: Effect of plant extract and alkaloid fraction on the growth parameters of weed *Cichorium intybus*

	W. somnifera		*H. muticus*	
CONC%	Germination% Water extract	Germination % alkaloid fraction	Germination % Water extract	Germination% alkaloid fraction
		Shoot length		
100 %	0	0	0	0
75%	0	0	28%	0
50%	84%	0	83%	0
25%	88%	0	94%	0
	Water extract %Germination %	Germination % alkaloid fraction	Water extract Germination %	Germination % alkaloid fraction %
		Root length		
100 %	0	0	0	0
75%	0	0	0	0
50%	68%	0	76%	0
25%	84%	0	88%	0

RESULTS AND DISCUSSION

The allophathic effect of plants (*W. somenifera* and *H. muticus)* were carried out by study the effect of aqueous extract and alkaloid fraction of the two plants on the germination of weeds (ciocherum,). The effects of aqueous extract and alkaloid fraction on germination of weeds after 7 days and 15 days in Petri dishes and in pots respectively are given in Table 1 and Table 2.Our findings demonstrated that two plant W. somnifera and *Hyoscyamus muticus* exhibited significant allelopathic activities in all parameters measured (germination, shoot and root length). As shown in Table 1&2, both alkaloid and aqueous extract significantly inhibited germination and reduced shoot and root length of *Cichorium intybus*. The result obtained were concentration dependent as increasing extract concentration significantly inhibited germination of *Cichorium intybus*. The highest inhibitory effect was shown by aqueous extract of W. somnifera than *H. muticus*

(100%) of high extract concentration(100%), while the alkaloid fraction of two plants have similar inhibition effect especially at high concentration while at low concentration the inhibition of germination decrease reach to (90% and 85%) for W. somnifera and H. muticus respectively as illustrated in Table 1. The effect of aqueous extracts and alkaloid fractions of two plants on root and shoot lengths on the studied weed were, like its effects on the germination indices, depending on concentration Table 2,. The root length of *Cichorium intybus*, was significantly reduced by aqueous plant extract and alkaloid fraction, which completely inhibited the root growth. The remaining concentrations of the extract completely inhibited the root growth of weed. In application of low concentration 25% of plant extract and 50% of alkaloid fraction showed significantly increased root length, whereas the higher concentrations induced significant gradual reductions in root length. The effect of extract on shoot length of the weed species exhibited the same pattern as that observed for root growth. At all concentrations, the plant extract inhibited the shoot elongation of , the degree of that inhibition increased gradually in parallel with increasing concentrations of the plant extracts and alkaloid fraction.Phytochemical analysis of aqueous extract of plants under study revealed the presence of many phytochemical class in two plant alkaloid, saponin, flavonoid and alkaloid as in Table 3.

GC-MS analysis of plants extract showed the presence of many bioactive compounds: As in Table 4, with their retention index (RI), structure formula and concentration. The results demonstrated the water extract of two plant contain the same phenolic compounds, 4-Hydroxy-3-methoxycinnamic acid (Ferulic acid), Methyl ferulate (,Hydroxy-6,7,8-trimethoxy-2,3-dimethyl-4H-chromen-4-one and mono benzylidene- glucose and Mandolic acid, also two plant contain some steroidal compounds, Sarreroside and Ergosta 5,22 dien,3-ol, acetate. There are some compounds are found in *W. somnifera* extract and not detected in H. muticus, Corynan-17-ol, 18, 19 di-dehydro10-methoxy-acetate and 3,9-Epoxypregnan-14 ol, 20-one,3,11,18-tri-acetoxy.the major compound in H. muticus are methyl ferulate (2.5%) and mono benzylidene- glucose (2.80). GC-MS analysis of alkaloid fractions of two plant showed the presence of many bioactive compounds As in Table 5,6 with their retention index (RI), structure formula and concentration. The results demonstrated the

Table 4: Phytochemical analysis of *Withania somnifera* and *Hyoscyamus muticus*

Compounds	*Withania somnifera*	RI	*Hyoscyamus muticus*
Methyl ferulate	0.08	604	2.5
4-Hydroxy-3-methoxycinnamic acid	0.06	615	0.08
4-hydroxy-mandonlic acid	0.28	604	0.65
psi.,.psi.-Carotene	0.35	501	0.2
Sarreroside	0.1	611	0.66
mono benzylidene- glucose	0.44	521	2.8
Hydroxy-6,7,8-trimethoxy-2,3-dimethyl-4H-chromen-4-one	0.33	420	0.44
Ergosta 5,22 dien, 3-ol, acetate	0.45	632	0.26
Cis Vaccenic acid	0.27	493	-
3,9-Epoxypregnan-14-ol, 20-one,3,11,18-triacetoxy	1.32	502	-
Corynan-17-ol,18,19-didehydro10-methoxy, acetate	0.31	404	-
α-D-Glucopyranoside	0.08	586	0.11

alkaloid fraction of two plant contain many same alkaloid compounds Morphinan4,5-epoxy3,6-diol, Strychane, 1-acetyl-20, β-hydroxy-16-methylene, Dasycarpidan1-methanol, Acetate and β-Hydroxyque brachamine with different concentration. Pterin-6-carboxylic acid are the major compound with concentration (2.5) in plant Withenia somnifera where it found in H. muticus with low concentration. Scopolamine and atropine are the major compounds in H.

Table 5: GC-MS- analysis of water extract of *Withania somnifera* and *Hyoscyamus muticus*

Compounds	*Hyoscyamus muticus*	*withania somnifera*	RI
Methyl ferulate	2.5	0.08	604
4-Hydroxy-3-methoxycinnamic acid	0.08	0.06	615
4-hydroxy-mandonlic acid	0.65	0.28	604
psi.,.psi.-Carotene	0.2	0.35	501
Sarreroside	0.66	0.1	611
mono benzylidene- glucose	2.8	0.44	521
Hydroxy-6,7,8-trimethoxy-2,3-dimethyl-4H-chromen-4-one	0.44	0.33	420
Ergosta 5,22 dien, 3-ol, acetate	0.26	0.45	632
Cis Vaccenic acid	-	0.27	493
3,9-Epoxypregnan-14-ol, 20-one,3,11,18-triacetoxy	-	1.32	502
Corynan-17-ol,18,19-didehydro10-methoxy, acetate	-	0.31	404
α-D-Glucopyranoside	0.11	0.08	586

Table 6: phytochemical analysis of Alkaloid fraction of *W. somnifera* and *H. muticus.*

Compounds	*Hyoscyamus muticus*	*withania somnifera*	RI
Methyl ferulate	2.5	0.08	604
4-Hydroxy-3-methoxycinnamic acid	0.08	0.06	615
4-hydroxy-mandonlic acid	0.65	0.28	604
psi.,.psi.-Carotene	0.2	0.35	501
Sarreroside	0.66	0.1	611
mono benzylidene- glucose	2.8	0.44	521
Hydroxy-6,7,8-trimethoxy-2,3-dimethyl-4H-chromen-4-one	0.44	0.33	420
Ergosta 5,22 dien, 3-ol, acetate	0.26	0.45	632
Cis Vaccenic acid	-	0.27	493
3,9-Epoxypregnan-14-ol, 20-one,3,11,18-triacetoxy	-	1.32	502
Corynan-17-ol,18,19-didehydro10-methoxy, acetate	-	0.31	404
α-D-Glucopyranoside	0.11	0.08	586

Table 7: Structure of alkaloid fraction in the two plants

Name of compounds	Structure
Strychane, 1-acetyl-20, α-hydroxy-16-methylene	
Curan-17-oic acid, 19,20-dihydroxy-, methyl ester	
β-Hydroxyque brachamin	
Kadain	

muticus with concentration (13.79% and 3.80%) respectively.

W. somnifera and *H. mutcus* are considered indicators allelopathic activity due to their ability of releasing allelophatic substance which has an affect on the germination of other plants. The allelophatic phenomena are found in gardening and agricultural fields since ancient times and this term mean biochemically mediated interaction in plants, many plant were noticed have this phenomena ,so in this study we need to use this phenomena to help us as natural pest side by using plant extract of *W. somnifera* and *H. mutcus* and their alkaloid fraction in germination inhibition of weed Ciochoruim instead of synthetic pest side and try to determine the chemical compound responsible for this inhibition. Secondary metabolites that exert allelopathy can be released in the form of volatile compounds, root exudates, above-ground plant leachates or plant litter [Duke, 2010]. Released allelochemicals are indeed subject to sorption on soil particles as well as chemical and microbial decomposition, (Kaur et al. 2009; Lankau 2010). (Macel et al. 2014), studied the role of many metabolite in six plant in allophathy effect the studied showed that plant chemistry is highly Specific-specific and diverse among both exotic and native species. Between class of metabolite have a role of allopathic effect are, phenolic compound, sesquiterpene, substituted alkaloid and flavonde in between phenolic compound have allophathy effect cinnamic acid derivatives (Lankau, 2010.), this are agree with our results which demonstrate the presence of cinnamic acid derivatives and methyl ferulate as constituents of two plant extracts. Also alkaloid compounds present in plant extract have a role in germination inhibition.

CONCLUSION

It is concluded that the aqueous extract and alkaloid fraction of *Withania somnifera* and H. mutcus showed an inhibitory allelophathic effect on the germination of wild chicory and the inhibitory effect depended on the concentration. Phytochemical analysis showed the plant contain different class of phytochemical compounds GC-Ms analysis of aqueous extract revealed the presence of hydroxyl cinnamic acid and methyl ferulate and other phenolic compound which may have a major role in germination inhibition also alkaloid fraction have many compounds which consider as alellochemical finally we can recommended by use these plant extracts as a pest side after further studies

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CONFLICT of INTEREST

The author declare any conflict of interest

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Amino acid Profile of the Watermelon, *Citrullus vulgaris* and Detection of its Antimicrobial Activity

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ABSTRACT

Seventeen free amino acids from the various parts of watermelon plant, including essential and non-essential amino acids were found by using Automatic Amino Acid Analyzer. In addition, the antimicrobial activity study of the susceptibility of watermelon (*Citrus vulgarisL*) against tested microorganisms was investigated. The identified amino acids included: phenylalanine, histidine, tryptophan, lysine, ornithine, arginine, aspartic acid, threonine, serine, glutamine, citrulline, alanine, valine, isoleucine and leucine. The essential amino acids (histidine, leucine, lysine, methionine, phenylalanine, tryosine, and valine ranged between 1.8%-3.6%, 5.5-9.1%, 3.7-6.7%, 0.4-1.1%, 4.1, 6.4%, 0.8-7.2% and 5.5-7.6% in the various parts of watermelon plant, respectively. There were notable variations between the various parts of watermelon plants in their contents of amino acids. However, the highest contents of most of the essential amino acids were concentrated in the leaves, while the highest contents of most of the non-essential amino acids were concentrated in the roots. The investigated amino acids had antibacterial and antifungal activities against the tested organisms.

KEY WORDS: AUTOMATIC AMINO ACID ANALYZER, HPCL, GLUTAMINE, VALINE, ISOLEUCINE.

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INTRODUCTION

Strains of the significant human pathogen have demonstrated expanded from all accessible traditional antibiotics (Aloush, 2006; Manchanda et al., 2010). A lot of systems have been engaged in bacterial resistance procedure including the mutation or enzymatic activation that results in the modified objective protein, gaining genes from other bacteria to express less susceptible focused on proteins, and furthermore obtain portable elements including transposons or plasmids (Baltzer and Brown 2011). In spite of nonstop endeavors, multiple drug resistance is as yet a genuine worldwide concern, especially a serious restorative danger to creating countries bringing about monetary weight and increment death rate. It is assessed that worldwide death because of antimicrobial resistance would reach to 10 million by 2050 (Wang et al., 2016). Antimicrobial peptides (AMPs have turned into a promising competitor and have gotten outstanding consideration as a novel class of antibiotics (Antimicrobial Resistance Benchmark 2018). They are peptides normally

delivered by all organisms including prokaryotes to human beings in response to foreign microbes and have a role in inborn explicit safeguard defense system and provide instant non-specific defense against infections. Amino acids are found either in the free state or as linear chains in peptides and proteins. There are 22 commonly occurring amino acids in proteins. Amino acid analysis has vital for the study of the composition of proteins, foods, and feedstuffs. Free amino acids are additionally determined in organic material, for example, plasma and urine, and in fruit juice and wine. When it is performed on a pure protein, amino acid analysis is equipped for distinguishing the protein (Hobohm et. al., 1994; Schegg et al., 1997), and the analysis is likewise utilized as an essential for Edman degradation and mass spectrometry and to determine the most reasonable enzymatic or chemical digestion technique for further investigation of the protein. Watermelon juice may give a novel source of the essential amino acid, arginine. Arginine is a precursor for nitric oxide, which has been appeared to lower blood pressure lessen blood thickening and ensure against myocardial localized necrosis and strokes (Collins et al., 2003; Cutrufello et.al., 2015; Fall et. al., 2019). Free amino acids such as phenylalanine, histidine, tryptophan, lysine, ornithine, arginine, aspartic acid, threonine, serine, glutamic acid, glutamine, citrulline, alanine, valine, isoleucine and leucine are accounted to have been extracted from watermelon and quantitatively analyzed (Tedesco et. al., 1984; Perkins-Veazie et. al., 2012; Jordan et al., 2019)). This study aimed to analyze the free amino profile of various parts of watermelon plant and detection of their antimicrobial activity.

MATERIALS AND METHODS

Amino Acid Analysis by Automatic Amino Acid Analyser S-433 Automatic Amino Acid Analyser S-433: The innovative automatic Amino Acid Analyzer S 433 combines the advantages of the classical ion exchange separation method with the modern technique of high performance liquid chromatography. The complete package of sophisticated instrumentation, a wide variety of preplaced and tested separation columns, combined with optimized ready-to-use buffer solutions and chemicals, create the right answer for any amino acid determination

High Performance Liquid Chromatography (HPLC): HPLC instrument consists of a reservoir of mobile phase, a pump, an injector, a separation column and a detector (Karen et al., 2019). Sykam chromatographs are modular systems optimized for customer's needs. Every single system can be configured to suit a certain application. Any existing Sykam HPLC can be upgraded by adding additional components.

ION Chromatography System - S 135

Ion Chromatography is one of the most important methods for the determination of alkaline, alkaline earth, transition metals, inorganic anions, sulphuric compound of different oxidation levels, organic acids and various tensides. Sykam S 135 is a compact system with modular setup. Even the basic system is designed for most sensitive anion analysis employing suppression of eluent conductivity.

Reagent organizer S 7131: The S 7131 Reagent Organizer is the optimal solutions for storing your solvents. The integrated gas supply with pressure regulator let you store the eluent under inert-gas pressure or for degassing purposes using Helium. The organizer can hold up to four eluent bottles which can be closed individually with integrated value bottle caps.

Conductivity Detector S 3115: The S 3115 Conductivity Detector's outstanding features are high background suppression, baseline stability, and signal linearity over a range of several decades. These characteristics become especially important when single column techniques are employed, e.g. for the determination of alkaline ions and alkaline earths.The HPLC Pump S 2100 is a compact eluent dosing system, upgradable from a high performance isocratic pump to a quaternary gradient pump with outstanding features.

Solvent Delivery System S 2100-Ex: The HPLC Pump S 2100 is a compact eluent dosing system, upgradable from a high performance isocratic pump to a quaternary gradient pump with outstanding features.

Sample preparation: For preparation of sample hydrolysate, 200 milligram of watermelon (roots, stems, leaves and three maturation stages of its fruit and seeds), was weighted in a hydrolysis tube to prepare hydrolysate sample. Then 5 ml of 6N HCl were added and the tube was tightly closed. The sample was incubated for 24 hours at 110°C. The sample was allowed to cool. 125 ml was filtered using 200 micro-liters of the filtrate were taken into another tube and evaporated at 140°C ovens for about 1 hour. 1 ml of diluting buffer was then added to the dried sample and transferred to amino acid analyzer vial and then injected for analysis. After the separation of the injected sample with a temperate cation separation column, ninhydrin was added continuously to the system. An integrated reagent dosing pump was responsible for the delivery of this reagent, while an external buffer pump was delivering the eluent. After adding ninhydrin, the eluent was lead through a high temperature reactor coil of about 16 m length. With a typical flow rate of 0.7 m1/min the buffer / ninhydrin mixture was stayed in the heated reactor for about 2 min. maximum absorption at 570 nm), while the secondary amino acids (have their maximum at 440 nm). After the reaction the mixture was lead through a dual-channel photometer where both wavelengths (570 nm and 440 nm) were measured continuously.

Tested microorganisms: The standard microorganisms used for this study were received from the Medicinal and Aromatic Plants Research Institute, National Centre for Research, Sudan and from the Department of Microbiology, Beijing University of Chemical Technology, China. They were *Bacillus subtilis*, B.s. (NCTC 8236, ACCC), designated as Gram *+ve, Staphylococcus aureus*, S.a. (ATCC 25923 & CVCC), designated as Gram *+ve, Escherichia coli, E. coli* (ATCC 25922, ACCC), designated as Gram -ve, and one fungus, *Candida albicans*, Ca.a. (ATCC 7596, CVCC), designated as Gram +ve.The microorganisms were produced a stock preparation containing a log-phase cell density of approximately 107 colony forming units (CFU)/ml as evaluated initially by measurements of the optical density at 600 nm.

Antibacterial Assay

Determination of antibacterial activity: After separation of amino acids in small pre-sterilized containers, they were taken for determination of antimicrobial activity. The antibacterial activity was assessed by the agar–well diffusion method (Kinsbury and Wagner, 1990). The inoculum size of each tested bacterium was adjusted to a suspension of 106 cells. The inoculum suspension was spread over a Mueller Hinton agar (MHA) plate to achieve confluent growth and allowed to dry. 10 mm-diameter wells were bored in the agar using a sterile cork borer. A 100 µL-aliquot of the reconstituted extract was placed into a well with standard Pasteur pipette and the plate was held for 1 hr at room temperature for diffusion of extract into the agar. Subsequently, the plate was incubated for 18 hr at 37°C. After incubation, the diameters of the zones of inhibition were measured to the nearest mm. Three replicates were performed and results were recorded.

RESULTS

Amino acid composition

Using the amino acid analyzer, the retention time and the amount of amino acid in the various parts of the watermelon plant as obtained from the chromatograms are indicted in Table (1) and in Figures (1 and 2). 17 essential and non-essential amino acid were identified. The identified amino acids included: phenylalanine, histidine, tryptophan, lysine, ornithine, arginine, aspartic acid, threonine, serine, glutamic acid, glutamine, citrulline, alanine, valine, isoleucine and leucine. The results also indicated that the highest amount of amino acids present were in this order, Glutamine (39.0%) then Ammonia (9.2%) and Arginine (7.7%) in the roots (SP_{1a}). The results indicated in Fig. 2 shows the identification of 17 essential and non-essential amino acids by using the amino acid analyzer to determine the retention time and the amount of amino acid in watermelon various parts. Results revealed that Glutamine has the highest amount among detected amino acids (39.0%) followed by Ammonia (9.2%) and Arginine (7.7%) in the plant roots (SP1a) (Fig. 2 -A). Also the highest amount of amino acids present the stems of watermelon (SP1b) was for Glutamine (21.90%) then Aspartic Acid (9.9%) and the Ammonia (8.2%) as indicated in Fig. 2 (B). On the other hand, Glutamine (19.5%) has the highest amount among amino acids present in the leaves of watermelon (SP1c), followed by Aspartic Acid (9.5%) and Leucine (9.1%) as shown in Fig. 2 (C). The results shown in Fig 2 (D) indicated that Glutamine Acid highest amount (20.4%) among amino acids in the green crust of watermelon (SP1d), followed by Arginine (12.3%) and Aspartic Acid (8.1%). Fig. 2 (E) also shows the identification of 17 essential and non-essential amino acids using the amino acid analyzer to determine the retention time and the amount of amino acids in white crust of watermelon. The results showed the that the highest amount found was Glutamic Acid (39.0%) followed by Ammonia (9.2%) and Arginine (7.7%) in the white crust of watermelon (SP1e). As for the watermelon kernel (SP1f), the highest amount present of was that of Glutamine Acid (18.7.0%), Arginine

Table 1. Essential and non-essential amino acids contents of various parts of watermelon plant

NO.	Amino acids	SP1a	SP1b	SP1c	SP1d	SP1e	SP1f	SP1h
1	Aspartic Acid	6.0	9.9	9.5	8.1	6.0	6.4	8.3
2	Threonine	2.8	4.0	4.7	3.8	2.8	3.0	3.3
3	Serine	1.8	2.9	3.0	3.7	1.8	2.5	2.7
4	Glutamine	39.0	21.9	19.5	20.4	39.0	18.7	22.7
5	Glycine	5.3	5.0	5.5	3.8	5.3	0.6	5.0
6	Alanine	2.6	6.6	6.1	4.0	-	7.7	5.1
7	Cysteine	-	0.1	0.7	0.2	2.6	0.5	1.0
8	Valine	5.1	7.6	7.2	6.9	5.1	7.3	5.9
9	Methionine	0.4	0.2	0.6	0.2	0.4	1.1	1.1
10	Isoleucine	4.3	5.7	5.7	5.3	4.3	5.8	4.7
11	Leucine	5.5	8.0	9.1	7.9	5.5	7.3	7.1
12	Tyrosine	0.8	0.5	7.2	0.7	0.8	1.0	1.3
13	Phenylalanine	4.1	5.6	6.4	5.6	4.1	5.4	6.0
14	Histidine	1.8	2.4	2.6	3.6	1.8	2.1	2.7
15	Lysine	3.7	6.4	6.4	6.7	3.7	2.5	4.4
16	Ammonia	9.2	8.2	4.3	6.9	9.2	11.9	4.0
17	Arginine	7.7	4.9	6.1	12.3	7.7	16.0	14.6

Watermelon plant = (SP1); SP1a= Roots; SP1b=Stem; SP1c= Leaves; SP1d = Green crust; SP1e = White crust; SP1f = kernel; SP1h = seed

Figure 1. Amino acid profile of the various parts of watermelon plant

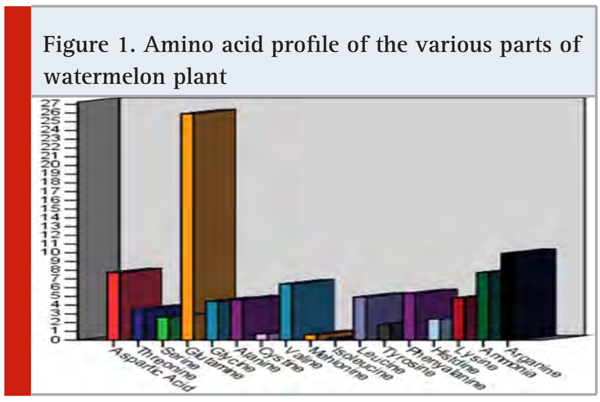


Figure 2. Free amino acids of watermelon (A) roots , stems (B), leaves (D), white crust (E),

A

B

C

D

E

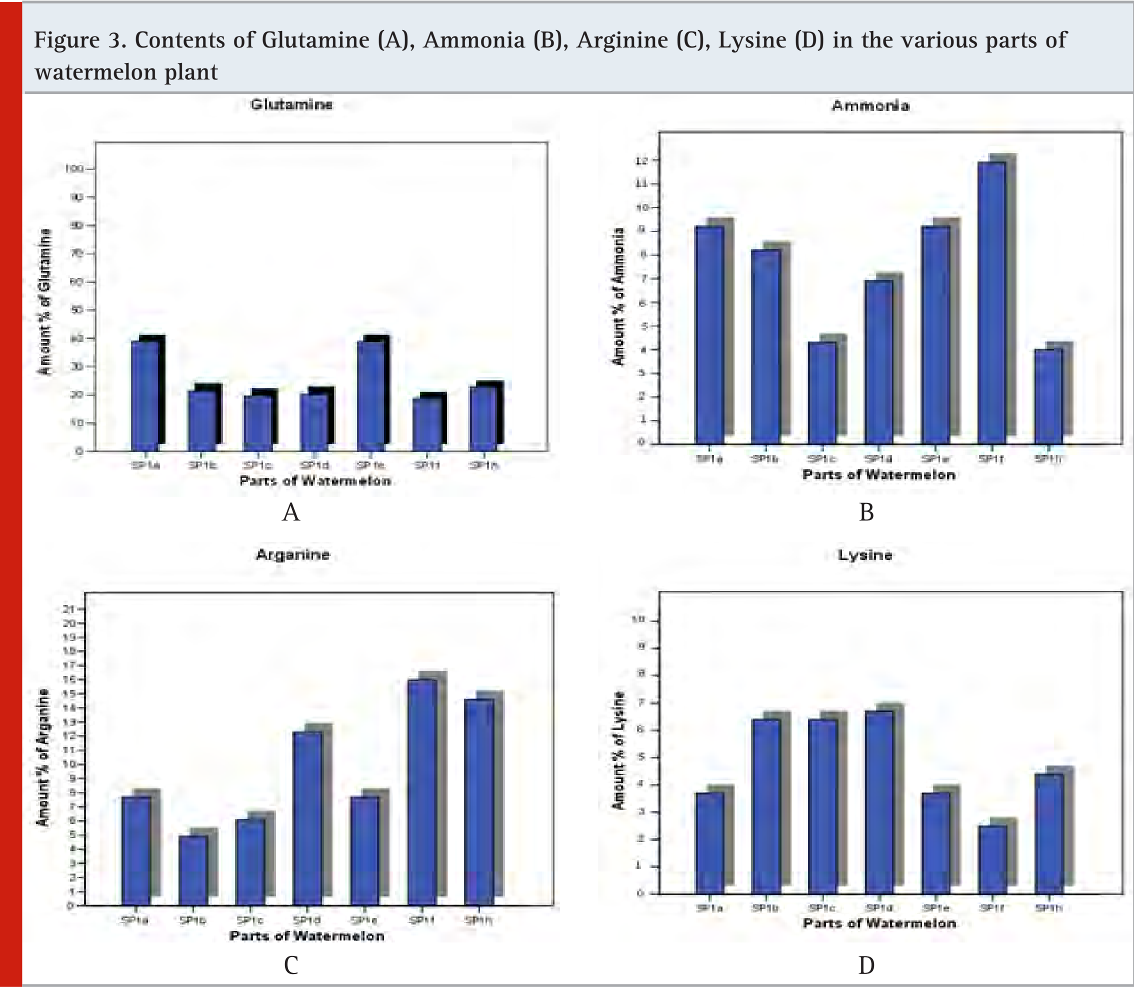


Figure 3. Contents of Glutamine (A), Ammonia (B), Arginine (C), Lysine (D) in the various parts of watermelon plant

(16.0%) and Ammonia (11%). The results showed that the highest amount of amino acids present in the seeds of watermelon (SP1h) was Glutamic Acid (22.7.0%), Arginine (14.6%) and Aspartic acid (8.3%).

Antimicrobial activity of the amino acids

The results of the antimicrobial activity of the free amino acids as shown in Fig. (4) shows that the investigated amino acids had antibacterial and antifungal activities against the tested organisms. However, the antimicrobial varies with the nature of amino acids, and the part of the plant and organisms. Fig. (4) also shows that the amino acid glutamine shows the highest antimicrobial activity against the fungus Candida albican which had the highest inhibition zone diameter (28 mm) followed by the bacteria *Staphylococcus aureus*, then E.coli and lowest activity was against Bacillus subtilis which had the lowest inhibition zone diameter (13 mm). The bacteria *Staphylococcus aureus* was highly inhibited by all detected amino acids with exception to serine and threonine. However, E.coli was inhibited by all detected amino acids with exception to serine. Bacillus subtilis was inhibited by all detected amino acids with exception to serine, lysine and cittruline. The fungus Candida albican was highly inhibited by all detected amino acids with exception to serine amino acid.

DISCUSSION

Amino acids contents: Amino acids are the building blocks of peptides and proteins. They possess two functional groups, the carboxylic acid group gives the acidic character, and the amino group provides the basic character. Proteins are

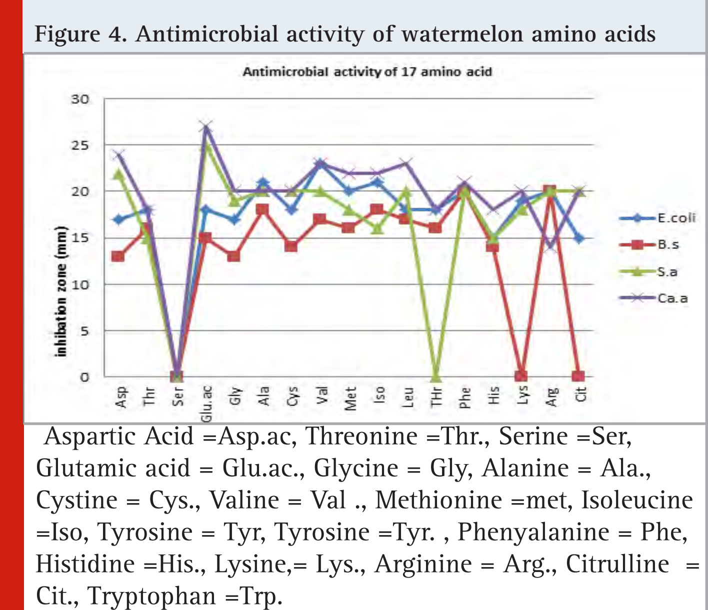
composed of different amino acids and hence the nutritional quality of a protein determined by the content, proportion, and availability of its amino acids (Becker, 2007). To date, more than 300 amino acids have been found in nature, of which 20 are engaged in protein synthesis and are known as proteinogenic amino acids. Proteinogenic amino acids exist in two structures: in a free state in physiological liquids (e.g., plasma, urine) and food (e.g., wine, beverage) and bound in peptides or proteins (Yu and Mou 2005).Table 1 shows the amino acid profile of watermelon various parts. This result suggested that the water melon plant parts were rich in amino acids, which might be served as good source of nutrition. Of 22 amino acids, almost 17 amino acids were present. However, some of the amino acids were present in lower concentration in comparison to that reported by FAO pattern (FAO 2018). The essential amino acids (Histidine, Leucine, Lysine, Methionine, Phenylalanine, Tryosine, and Valine ranged between 1.8%-3.6%, 5.5-9.1%, 3.7-6.7%, 0.4-1.1%, 4.1, 6.4%, 0.8-7.2% and 5.5-7.6% in the various parts of watermelon plant, respectively.

Valine essential amino acid was the dominant essential amino acid in all watermelon plant parts. Moreover, watermelon stem contained the highest value, while both the root and white kernel contained the least value. On the other hand, Methionine amino acid was the least in concentration among all essential amino acids in all watermelon plant. Nonessential amino acids (Alanine, Aspartic acid, Glutamine, and Serine) ranged between 1.8 – 39% for all watermelon plant parts. Glutamine was the dominant non-essential amino acids in all watermelon parts. Moreover, it was the most dominant amino acid investigated in this study. Most of the amino acid values are comparable with those of most vegetable protein determined by many investigators (El- Adawy et. al., 2001; Mune et al., 2011; Ogunladeet al, 2011; Sánchez- Vioque et. al., 1999). Generally the average percentage of nonessential amino acids was higher in concentration than essential amino acids in watermelon plant parts. In comparison, Usman et al. (2010) revealed the prevalence of glutamic acid and aspartic acid in watermelon seeds. This suggests variety happens as indicated by genotype and the geographical and environmental conditions in which watermelons are developed. Glutamine is an important amino acid with numerous capacities in the body. It is a building block of protein and critical part of the immune system. Likewise, glutamine has an extraordinary role in intestinal health. Your body naturally produces this amino acid, and it is also found in numerous foods. There were notable variations between the various parts of watermelon plants in their contents of amino acids (Table 1).

However, the highest contents of most of the essential amino acids were concentrated in the leaves (SP1a), while the highest contents of most of the non-essential amino acids were concentrated in the roots (SP1b). Watermelon plant = (SP1); SP1a= Roots; SP1b=Stem; SP1c= Leaves; SP1d = Green crust; SP1e = White crust; SP1f = kernel; SP1h = seed. A high- quality protein contains essential amino acids in ratios proportionate with human needs. This can be dictated by comparing the amino acid contents of different proteins with the FAO reference pattern. The FAO reference pattern dependent on the essential amino acid requirements of young children (1–2 years) is viewed the preferred reference protein (Andini et. al., 2013). Methionine and Tyrosine were found in limited amount for most of the watermelon plant parts, however, this shortage might be explained by their denaturization during analysis or their values are very limited in the plant. To compensate this limitation in watermelon, additional Utilization of animal or plant proteins, for example, milk, egg, lentils, and heartbeats are profoundly prescribed (Silva et al. 2014).

Antimicrobial activity: Antimicrobial peptide (AMPs) is a part of inborn safeguard framework in most multicellular life forms, from people to plants to insects. To date more than 2200 common or synthetic AMPs have been accounted for through the antimicrobial peptide database (APD) (Silva et al. 2014). Antimicrobial peptides (AMPs) are commonly composed of short sequences of 10–100 amino acids deposits and profoundly and membrane active (Reddy et al. 2004). AMPs have a wide range of antibacterial, antifungal, antiviral, and anti-tumor activity at low concentrations. In

Figure 4. Antimicrobial activity of watermelon amino acids



Aspartic Acid =Asp.ac, Threonine =Thr., Serine =Ser, Glutamic acid = Glu.ac., Glycine = Gly, Alanine = Ala., Cystine = Cys., Valine = Val ., Methionine =met, Isoleucine =Iso, Tyrosine = Tyr, Tyrosine =Tyr. , Phenyalanine = Phe, Histidine =His., Lysine,= Lys., Arginine = Arg., Citrulline = Cit., Tryptophan =Trp.

contrast to traditional antibiotics, antimicrobial peptides can direct the host resistant immune system and kill bacteria directly (Gottler and Ramamoorthy 2009). Moreover, other biological activities of AMPs have also been described as following: neutralization of endotoxins, immune-modulating properties, chemokine-like activities, and induction of both angiogenesis and wound repair (Guaní-Guerra et al. 2010). It has been reported that Gram-positive bacteria are known to be more susceptible to amino acids complexes than gram-negative bacteria. The weak antibacterial activity against gram negative bacteria was attributed to the existence of an external film which stances hydrophilic polysaccharides chains as a a boundary to the amino acids complexes.

It has also been reported that the antibacterial action of a complex is affected by its dependability. The lower strength of the amino acid complex, the more prominent is the antibacterial action. This is likely because they have more free ions in the solution, which can improve the cooperative association between the metal particles and the ligands, (Marcu et al., 2008; Stanila et al., 2007). The variety in the adequacy of various compounds against various organisms depends either on the impermeability of the cells of the microorganisms or the distinction in ribosomes of microbial cells. It has additionally been suggested that concentration assumes a fundamental role in expanding the level of inhibition; as the concentration increases, the activity increments as it was accounted for likewise in the present cases (El-Wahab et al., 2005). In this context further investigations are expected to understanding the antibacterial or antifungal mechanism Antimicrobial peptides which are composted of a great number of amino acids have a broad spectrum of activity against a wide range of microorganism including Gram negative and Gram positive bacteria, fungi, parasites and viruses (Brown et al., 2007; Hancock (2001); Andrea and Rivas (1998); Bradshaw 2003).

CONCLUSION

There are a total of 17 free amino cids that were found in all organs of watermelon plant, out of which glutamic acid, arginine and aspartic acids were found to be in higher concentration in all organs of the plant. However, the highest contents of most of the essential amino acids were concentrated in the leaves, while the highest contents of most of the non-essential amino acids were concentrated in the roots. The results of antimicrobial activity tests, show that amino acids complexes have inhibitory effect against the bacteria: *Staphylococcus aureus*, *Escherichia*

coli, and the fungus, *Candida albicans*, and less efficient against Bacillus subtilis. Studies regarding the mode of action for the amino acids in the microbial bacterial cell should be investigated, since these substances are natural, their hazardous potential is lower when compared with other products.

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Influence of Gingival Retraction Agents and Adhesive Bonding Regime on the Bond Strength of Composite to Dentin

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ABSTRACT

The aim of the present study was to evaluate the influence of retraction agents (Expasyl and viscostat) and bonding regimes [Total-etch, Self-etch and Er,Cr:YSGG (ECL) laser] on the shear bond strength (SBS) of resin composite to dentin. One hundred and eighty human mo-lars were prepared for exposure of occlusal dentin. Based on retraction agent treatments of dentin, teeth were equally divided into three main groups, A: Expasyl, B: Viscostat, C: No treatment (Control) (n=60). After dentin cleaning, dentin specimens in each group were bonded to resin composite (Tetric N-Ceram) using three different bonding regimes, 1: Etch & rinse, 2: ECL and 3: Self-etch. These combinations resulted in none study groups (n=20). Bonded specimens were exposed to shear bond strength testing using universal testing ma-chine under a standard load applied at crosshead speed of 1mm/min. Failure mode of the fractured specimens were assessed using stereomicroscope. Data was analysed using analysis of variance and Tukey-Kramer multiple comparisons test.The maximum bond strength was displayed by group C1 (Etch & rinse + Tetric-N- Bond) (24.54± 3.55 MPa) and lowest bond scores were in group B3 (Viscostat + Clearfill SE) (14.52±2.23 MPa). SBS was significantly higher in control s as compared to specimens exposed to Expasyl and viscostat in all corre-sponding groups ($p<0.05$). Different bonding techniques (Etch-rinse, ECL and Self-etch) showed comparable SBS outcomes in all corresponding hemostatic agent groups ($p>0.05$). Most common failure mode among the specimens of study groups was adhesive. The use of haemostatic agents compromised bond integrity of resin composite to dentin irrespective of the bonding regime employed. Post haemostatic agent application, Er,Cr:YSGG, etch-rinse and self-etch conditioning techniques showed comparable bond strength outcomes of resin to dentin.

KEY WORDS: EXPASYL; VISCOSTAT; ETHC-RINSE, SELF-ETCH; BOND STRENGTH; ER,CR:YSGG LASER.

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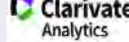

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INTRODUCTION

Dental operative procedures when contaminated with saliva and blood, compromises resin bonding to dentin (Cacciafesta et al., 2004). Rubber dam and other isolation devices, act as a physical barrier against blood and salivary contamination but, its utilization turns out to be impractical and unfeasible in certain clinical situations. Consequently, use of haemostatic agents and retraction cord materials gain importance in these clinical scenarios. These clinical circumstances may range from Class V cavity preparation, impression taking to intra-sulcular restorations and crown cementation (Cochran et al., 1989 Madarati et al 2018). Haemostatic agents are commonly employed for isolation and soft tissue management proce-dure for multiple dental applications. Amongst them, viscostat comprised of 20% ferric sul-phate and Expasyl, containing solution of aluminium chloride are popular (Saati et al., 2018).

Expasyl is formulated in a way to curtail the damage of healthy periodontium avoiding gingi-val recession or bone resorption. Moreover, viscostat having a pH of ~1.0, stops bleeding, provides isolation and dry field in chairside intra-oral practices. It is gentle on hard and soft tissues and eliminates sulcular fluid contamination for optimal bonding (Ahmad et al., 2015). However, evidence suggests that aluminium chloride and ferric sulphate based haemostatic agents induce changes in dentin and enamel and alters the quality of adhesive hybrid layer, hence compromising the bond quality and strength (Giannini et al., 2015). Different bonding system have been developed to improve bond strength, reduce microleak-age, decrease chair side time and minimize contamination (Giannini et al., 2015; Santos et al., 2014). Both the systems have their advantage and disadvantage and their selection varies de-pending on different clinical scenarios (Migliau, 2017; Santos et al., 2014). Alternatively, laser in the form of Er,Cr:YSGG (ECL) to condition dentin, enamel and lithium disilicate ceramics (LDC) has exhibited exceptional outcomes (Alkhudhairy et al., 2019c, 2019a, 2019b; F. Alkhudhairy et al., 2018a, 2018b; Vohra et al., 2019, 2018).

ECL works on the principal of ablation and destruction of dentinal surface by hydration of organic component and water from the dentinal tissues working at a wavelength of 2. 78mm. This method of dentin condi-tioning has gained exceptional appraisal due to less technique sensitivity, predictable out-come, controlled conditioning and safety (Alkhudhairy et al., 2019c, 2019a). To our knowledge from indexed literature, evidence related to ECL as dentin conditioner af-ter using different haemostatic material is scarce. Moreover, limited evidence on comparative studies between self-etch and total etch bonding materials after using haemostatic agents is available. It is hypothesized that pre-application of haemostatic agent and dentin conditioning with ECL will exhibit comparable outcomes to dentin conditioned with etch and rinse after haemostatic agent application. Therefore, the aim of the study was to evaluate the influence of haemostatic agents (Expasyl and viscostat) and bonding regimes [Total-etch, Self-etch and Er,Cr:YSGG (ECL) laser] on the shear bond strength (SBS) of resin composite to dentin.

MATERIAL AND METHODS

One hundred and eighty non-fractured, permanent non-carious, intact, third molars were iso-lated and cleaned from inorganic remnants and debris with the help of periodontal curette and scaler (Superior Instruments Co, New York, USA). The teeth were stored in thymol solution (0.5%) for two weeks to disinfect and then were kept in distilled water at 4°C. Within the sections of polyvinyl pipes (8mm diameter), teeth were placed vertically in acrylic resin (Ortho-Jet, Lang Dental MFG, IL, USA) up to the cementoenamel junction. To maintain uniformity the dentin surface was exposed by cutting with Diamond saw on slow speed machine and polished with silicon carbide paper (Buehler, Lake Bluff, IL, USA) under water irrigation for 10 sec at 250rpm using a polishing machine (Aropol 2V, Arotec). Based on gingival retraction materials used, samples were divided into three groups.

Group A (n=60): Application of Expasyl (Acteon Pharma) gingival retraction paste. Five minutes after application the material became visible owing to its colour change. The paste was washed off by air water spray for 30 sec and air dried.

Group B(n=60): ViscoStat (Ultradent Product, USA) was applied on dentin with a micro-brush and removed with running water spray after two minute and air dried.

Group C (n=60): (No treatment control): There was

no application of gingival retraction ma-terial (Table 1). Now based on surface conditioning protocol each group was further classified into three sub-groups as A1, A2, A3; B1, B2, B3; C1, C2, C3 with 20 samples in each group.

Subgroup A1, B1 and C1: Samples were conditioned with 35% phosphoric acid (Ultra-Etch; Ultradent Products, Inc., South Jordan, UT, USA) for 15sec and rinsed for 10sec. A universal bonding agent (Tetric N-Bond Universal, Ivoclar-Vivadent) was applied and light cured (Bluephase G2, Ivoclar,Vivadent) for 10 sec.

Subgroup A2, B2 and C2: Dentinal surface of each given sample was conditioned by ECL (Waterlase C100, BioLase Tech Inc., California, USA) power 4.5W and frequency 30Hz in a non-contact mode from a distance 2mm using tip (MZ=8) for a duration of 60 sec. after con-ditioning procedure a universal bong agent was applied as discussed in previous subgroups.

Subgroup A3, B3 and C3: Clearfill SE (two step self-etch) was applied on all samples. Priming for 20 seconds (no mixing was done). Bond application and light curing for 10 sec using Bluephase G2 (Ivoclar,Vivadent).Surface conditioning of dentin was followed by ap-plication of Tetric N-Ceram (Ivoclar Vivadent). The bonding of composite was performed in cylindrical block of 4.5mm diameter and 2.45mm height. The application of composite was in line with the instructions of the manufacturer. To simulate oral conditions thermocycling of all the samples was done between 5°C to 55°C for 10000 cycles.

Shear Bond Strength (SBS) testing: In a Universal testing machine (Instron Santam, model STM-20, Riyadh, KSA) 20 samples from all nine subgroups were placed under known static loads at a cross head speed of 1mm/min. The force applied by universal testing machine was kept parallel to bonded surface. The force to debond sample was calculated in megapascal (MPa).

Failure mode Analysis: Two examiners performed fracture analysis using stereomicroscope at 40x magnification (SZX7, Olympus, Hamburg, Germany). Failure mode was classified into three categories i.e., admixed, cohesive, adhesive. Statistical Analysis:Data was tabulated using statistical program for social science (SPSS version 21, Inc., Chicago, US) for bond strength

Table 1. Materials used in this study

Material	Composition	Batch #	Manufacturer
Viscostat	20% Ferric Sulfate equivalent solution	B57QB	Ultradent
Expasyl	Aluminum chloride 15% equivalent solution	3293	Acteon Pharma
Tetric N-Ceram	Dimethacrylates, Polymer filler, barium glass filler, Initiator, stabilizer pigments.	S14434	Ivoclar Vivadent
Tetric-N-Bond	BISGMA, 2-hydroxyethyl methacrylate, phosphonic acid acrylate, Urethane Dimethacrylate	584747	Ivoclar, Vivadent
Clear fill SE Bond	MDP, HEMA, Water, Camphorquinone, NN-di-ethanol p-touloudine	125478925	Kuraray America Inc

testing outcomes. Normality of the data obtained was evaluated using Kolmogorov-Smirnov test. Using analysis of variance (ANOVA) and Tukey's post hoc test at a significance level of p = 0.05 means and standard deviations (SD) were compared.

RESULTS AND DISCUSSION

Data in the present study were normally distributed. Table 2 demonstrates SBS values among experimental groups. The maximum bond strength was displayed by group C1 (Etch & rinse + Tetric-N- Bond) (24.54± 3.55 MPa) and lowest bond scores were in group B3 (Viscostat + Clearfill SE) (14.52±2.23 MPa). Different superscript alphabets denote statistically significant difference within same row and column.! Showing significant difference among study group (ANOVA). Based on application of hemostatic agents, bond strength values among subgroups A1 (17.55±3.85) and B1 (16.54±2.46) were found to be comparable p>0.01. Moreover, subgroup C1 (24.54± 3.55) exhibited significantly higher SBS compared to A1 and B1 (p<0.01). Simi-larly, SBS among A2 (16.21±3.41) and B2 (15.25±3.27) specimens were comparable (p>0.05), however lower than C2 (22.25± 4.78) specimens. Furthermore, subgroup C3 (21.35±3.24) showed higher SBS compared to A3 (15.62±2.39) and B3 (14.52±2.23) (p<0.01).

Overall, application of hemostatic agents (Expasyl and viscostat) significantly re-duced SBS values. Moreover, based on dentin conditioning, bond strength scores among groups C1 (Etch & rinse + Tetric-N- Bond) (24.54± 3.55), C2 (ECL + Tetric -N- Bond) (22.25± 4.78) and C3 (Clearfill SE) (21.35±3.24) were comparable (p >0.05).. Overall, different conditioning re-gimes exhibited comparable outcomes for SBS values among the specimens of groups A1,A2,A3,B1,B2,B3 (p>0.05).. For bond strength values, analysis of variance (ANOVA) showed significant difference among all study groups (p<0.05). Failure modes observed among the de-bonded specimens are presented in table 3. Most of the failures in viscostat group were adhesive (B1 55%), (B2 65%) and (B3 80%). Moreover, in lased specimens ad-mixed failure was more frequent (A2 50%) and (C2 70%). Cohesive failure was common among specimens of groups A1 and C1. Overall, in all groups adhesive failures were com-monly observed.

The present study was based on the hypothesis that pre-application of haemostatic agent and conditioning of dentin with ECL will exhibit comparable outcomes to dentin, preconditioned with total etch and rinse after application of haemostatic agents. Subsequently, the hypothesis was rejected as application of haemostatic agent in combination with ECL and etch & rinse compromised bond strength values. Control group specimen without application of haemo-static agents displayed better bond integrity compared to specimens treated with haemostatic agents. In the present study bond integrity was evaluated using universal testing machine. The meth-od has low technique sensitivity and gives comparative analysis between groups. Moreover, this reliable and precise test is homogenized, standardized

Table 2. Comparison of means and SD for bond strength values among study groups using ANOVA and Tukey multiple comparisons test.

Experimental group	Etch & rinse + Tetric-N- Bond (1)	ECL + Tetric -N- Bond (2)	Clearfill SE (self-etch) (3)	P value!
Expasyl (A)	17.55±3.85 a	16.21±3.41a	15.62±2.39 a	<0.01
Viscostat (B)	16.54±2.46 a	15.25±3.27 a	14.52±2.23 a	
No treatment Control (C)	24.54± 3.55 b	22.25± 4.78 b	21.35±3.24 b	

A1: Expasyl + Etch & rinse + Tetric-N- Bon, A2: Expasyl + ECL + Tetric -N- Bond, A3: Expasyl + Clearfill SE (2 step self-etch) B1: Viscostat + Etch & rinse + Tetric-N-Bond, B2: Viscostat + ECL + Tetric -N- Bond, B3: Viscostat + Clearfill SE (2 step self-etch),C1: Etch & rinse + Tetric-N- Bond, C2: ECL + Tetric -N- Bond, C3: Clearfill SE (2 step self-etch)

and is consistent with other studies (Sirisha et al., 2014a, 2014b). Adhesives are sensitive to moisture and blood contaminants. For optimum bond strength operating field free of moisture is inevitable (Ahmad et al., 2015). Presently, haemostatic material Viscostat, containing 20% ferric sulphate displayed the lowest SBS scores (14.52±2.23) compared to all other experimental groups, though the results were statistically insignificant. These findings were found to be in harmony with studies by (Unlu et al., 2016 and Ebrahimi et al., 2013). A possible explanation to low bond scores can be attributed to viscous nature of viscostat making its removal difficult after etch and rinse and ECL conditioning of dentin (Pucci et al., 2016). Moreover, the composition of viscostat promotes coagulation of proteins in dentine resulting in poor penetration of adhesives. Furthermore, a study, by Kimmes et al., (2006) proclaims viscostat application on dentin does not alter SBS values. Different types of study methodologies, nature of adhesives, form of dentin (superficial and deep) and curing time may attribute to varied results.

Amongst the different conditioning regimes used in the present study, Clearfill SE (2 step self-etch) displayed low bond integrity scores with both haemostatic agents Expasyl (15.62±2.39) and Viscostat (14.52±1.23). Clearfill SE comprises of maleic acid (weak acid) which is not able to penetrate the haemostatic material and deeper areas of dentin, compro-mising bond values (Margvelashvili et al., 2010). Moreover, low pH of haemostatic agents (between 1.5 to 3.86) and poor penetration of HEMA monomer in Clearfill SE obturates and plugs the dental tubules forming amorphous layer over the dentin and altering the quality of smear layer hence hindering the penetration of 2 step self-adhesive (Ayo-Yusuf et al., 2005; Margvelashvili et al., 2010).

Table 3. Modes of failure among different experimental groups

Experimental groups	Adhesive (%)	Cohesive (%)	Admixed (%)
A1	20	70	10
A2	20	30	50
A3	70	20	10
B1	55	25	20
B2	65	15	20
B3	80	10	10
C1	20	50	30
C2	25	5	70
C3	5	30	65

A1: Expasyl + Etch & rinse + Tetric-N- Bon, A2: Expasyl + ECL + Tetric -N- Bond, A3: Expasyl + Clearfill SE (2 step self-etch) B1: Viscostat + Etch & rinse + Tetric-N-Bond, B2: Viscostat + ECL + Tetric -N- Bond, B3: Viscostat + Clearfill SE (2 step self-etch),C1: Etch & rinse + Tetric-N- Bond, C2: ECL + Tetric -N- Bond, C3: Clearfill SE (2 step self-etch)

To our surprise conditioning of dentin with ECL and etch and rinse showed comparable SBS outcome both with and without haemostatic agents. ECL when used at 4.5W and 30Hz working at a wavelength of 2780nm exhibits strong affinity with hydroxyapatite and water, is well absorbed by the dentin structure itself resulting in increase size in dental tubule orifice, forming irregular rugged appearance free from smear layer and disposal of both organic and inorganic structure. Hence, improving dentin permeability and receptiveness to bond (Alkhudhairy et al., 2019c; Alkhudhairy et al., 2018). Moreover, conventional etch and rinse method of dentin conditioning. Showed highest SBS values amongst all groups both in the presence (17.55±3.85) (16.54±2.46) and absence (24.54± 3.55) of haemostatic agents. A probable description to this outcome is 37% phosphoric acids in etch and rinse, this creates micro porosities and better mechanical retention between dentin surface and adhesive pro-moting better fluid movement. Furthermore, phosphoric acid completely removes smear layer and peritubular dentin for better bond integrity (Bertolotti, 1991; Turp et al., 2013).

Further-more, the authors speculate that ECL and etch and rinse conditioning along with added ad-vantage of HEMA in ethanol based Tetric-N-bond enhances wettability with its low hydro-philic nature promoting adhesion (Kumari et al., 2015). Interestingly, Expasyl haemostatic agent, showed better bond integrity compared to viscostat. Primarily, reason for better result is less acidic pH of expasyl (pH-3.86) compared to viscostat (pH-1.78). Hence, expasyl being gentler on the dentin (Tarighi and Khoroushi, 2014). Moreover, study by Lahoti, (2016) ad-vocates that expasyl does not alter the smear layer resulting in better SBS values. Further-more, a study by Harnirattisai

et al., (2009) proclaimed that expasyl on dentine causes no change in dentinal contents compared to viscostat resulting in better bond quality.

Majority of the failures in Clearfill SE groups in combination with expasyl and viscostat were adhesive. The type of failure is favourable as it results in less iatrogenic damage to the tooth structure (Henkin et al., 2016). Likewise, this failure type corresponds to low SBS in self etch group. Similarly, in lased and only Clearfill SE group admixed failure type was frequent. The type of failure corresponds to high SBS values. Admixed type of failure results from stress and fracture within the material itself. Factors, contributing to this failure type may include type of debonding procedure, lateral forces and nature of conditioning pattern (Almoammar, 2019).

Likewise, high incidence of cohesive bond failure was experienced in groups condi-tioned with etch and rinse. Application of haemostatic agents not just results in chemical interaction but also physical topographical modifications in dentin. However, this was not assessed in the present study. In addition, contemporary resin based bioactive materials are able to chemically interact with dentinal surface and continuously allow for release and recharge of ions, potentially improv-ing bond integrity. Therefore, the use of these materials as an alternate to conventional resin for restorations in combination with haemostatic agents should be investigated. Furthermore, scanning electron microscopy and surface profilometry studies on dentinal surfaces after ap-plication of haemostatic agent and ECL to develop insights in topographical changes are rec-ommended.

CONCLUSION

The use of haemostatic agents compromised bond integrity of resin composite to dentin irre-spective of the bonding regime employed. Post haemostatic agent application, Er,Cr:YSGG, etch-rinse and self-etch conditioning techniques showed comparable bond strength outcomes of resin to dentin.

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The Occlusal Status of 8 to 10 Years Old Vietnamese Children: A Cross-Sectional Study

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ABSTRACT

Malocclusion can cause frequent bites of cheeks or tongue, cosmetic problems, irritability or chewing, speech problems or development of breath thus affecting the quality of life in general. The purpose of this study was to estimate the prevalence of different degrees of deviation related to oral complications in children aged 8-10 years. A cross-sectional study was conducted on 686 children aged 8-10 years after getting parental consent. Vietnamese children are selected from certain cities by using proportional allocation techniques by dividing these cities into districts where children are selected from the center and the garden is proportional high school.Based on molar relationships, the incidence of malaria in children aged 8-10 years (children with mixed teeth) was 35.42%, of which 56.12% had a level II division with or without subdivision and 31.20% have class III with or without zoning. The remaining 12.68% of children have different types of malaria including type II and II and type III malformations. Common bites are reported in 34.11% of children, other types of bites include open bites, deep bites, bites from one side to the other are recorded respectively 47.23%, 15.45%, 3.21% in children. This study shows that more than one third of Vietnamese children are affected by different types of errors and deviations. Important associations were discovered between the age of children and the prevalence of malaria and crowding.

KEY WORDS: CHILDREN; CROWDING; DIASTEMA; MALOCCLUSION; PREVALENCE; VIETNAM.

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INTRODUCTION

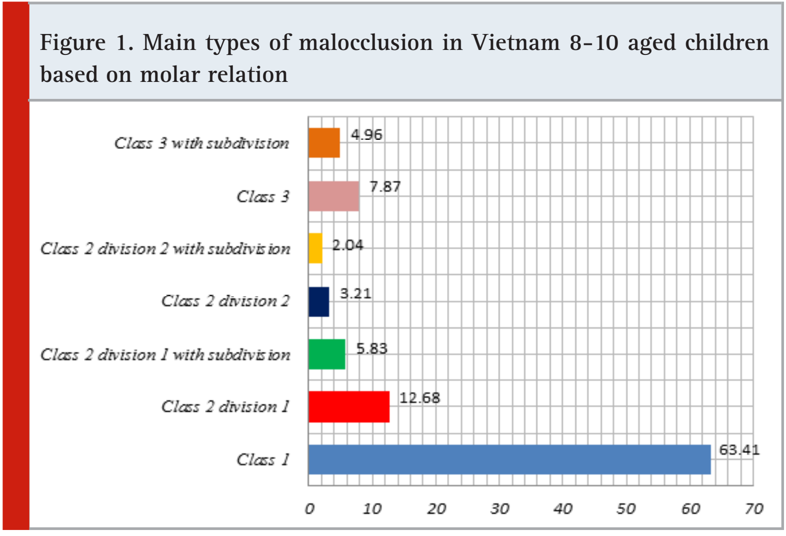
Research has shown that the occlusion of a tooth is important in determining the occlusion in permanent teeth (Radnzic,1988). Malocclusion treatment is a big challenge for dental professionals because of the complex and expensive treatment, so the focus is on early diagnosis and prevention. Determine the incidence of malaria in children, leading to early diagnosis and setting up the need for social treatment to help orthodontic doctors and pediatricians make appropriate treatment plans and preventive planning and prevent (Almasri 2014). A number of published studies on major dental obstruction in many countries (Clinch 2007; Jensen et al, 1957; Boyko 1968; Otuyemi et al, 1997; Abu Alhaija & Qudeimat 2003; Meer et al, 2016). However in Vietnam, the number of published documents is limited. The exclusion in primary dentistry has been found to be affected by many types of malocclusion, an old study found that as high as three-quarters of 3-year-old children had a relationship of occlusion (Chapman 1935).

The clinic reported a 43% incidence in asymmetrical molar teeth relationships in 61 children (Clinch 1951). Similarly, other studies of India, Saudi Arabian and Israel have reported more than half of children with a final relationship with incidences of 52% and 68% respectively (Kaufman & Koyoumdjisky 1967; Abumelha et al, 2018). In Arab children, a study showed that the rate of end-to-end contact was as high as 80% in 3-5 year olds (Farsi & Salama 1996). In addition, other defects such as mesial and distal steps have been reported in Saudi children. Malocclusion refers to a number of possible conditions. It may be an improper connection of the teeth, or a change in the appearance of the face. Malocclusion can cause frequent bites of the cheek or tongue inside, cosmetic problems, discomfort or chewing, voice problems or mouth breathing development thus affecting the quality of life in general (Abumelha et al, 2018). This study aims to estimate the prevalence of different levels of malocclusion related to oral complications in children aged 8-10 years. The findings of this study can help us understand the need for social treatment and help all relevant parties to develop appropriate prevention and treatment plans.

MATERIAL AND METHODS

A cross-sectional study was performed on 686 children aged 8-10 years after receiving consent from parents. Vietnam children are selected from certain cities by using proportional allocation techniques by dividing these cities into districts where children are selected from the center and the garden with attendance rates high. Disposable pen, pencil and ruler test kits were used to evaluate the occlusion status of the study participants. Subjects were classified as having Type I, Type II and Type II malaria by using the

Figure 1. Main types of malocclusion in Vietnam 8-10 aged children based on molar relation



angle classification of the molar relationship. Inclusion / exclusion criteria: All children aged 8 to 10 years, ready to participate in the study were included. Children with craniofacial deformities, those who are medically injured and have undergone any orthodontic treatment are excluded from the study.Diagnosis has been reported in the data collection table along with the demographic data of the subjects. Therefore, the collected data was entered into Microsoft Excel spreadsheet and included in statistical analysis by SPSS version 20.0 (social science statistical package). Before conducting research, moral clearance was taken from the dental college Ho Chi Minh city.

RESULTS AND DISCUSSION

The total sample of 686 children aged 8-10 years was included in this study, of which 80% were female and 20% were male. More than half of the children are from Ho Chi Minh City while about one third of children are selected from Hanoi City (Table 1 to 3). Based on molar relationships, the incidence of malaria in Vietnamese children aged 8-10 (mixed teeth) is 35.42%, representing 243 children, of which 56.12% have class I. or without subdivision and 31.20% have category III with or without subdivision. 12.68% of the remaining children have malformations with different types of malaria including Class II subdivision II and combination of class II and III malformations (Figure 1). In the present study, the antero-posterior molar relationship showed the prevalence of 35.42% of malocclusion in Vietnam children aged 8-10. Similarly, Alzubair and Ghandour found 30.6% prevalence of discrepancies in antero-posterior molar relationship in 12-yearage Yemeni children (Al-Zubair & Ghandour, 2014). Abumelha et al, found 35% prevalence of discrepancies in antero-posterior molar relationship in 6-12-year-age Saudi Arabian children (Abumelha, et al, 2018). Johannsdottir et al, found 33% and 36% prevalence of malocclusions in 6-year-age children (Johannsdottir et al, 1997). A study among Lithuanian 7-15 years aged children found that only 15.3% had normal occlusion (Sidlauskas & Lopatiene, 2009). Thailander found that malocclusions found in 88% of 5-7 years old children. This difference in malocclusion prevalence can be attributed to the genetic factors and different criteria used to define malocclusions. Several studies have reported a high prevalence of malocclusion among school children which varied between populations in Saudi Arabia, the seeking for orthodontic treatment has been grown in the last decade (Hassan, 2006).

In the present study, 23.76% of studied children had class II and 12.83% had class III, Abumelha et al, found 22.8% of studied children had class II and 12.2% had class III (Abumelha et al, 2018). And while Johannsdottir et al, found 27% of boys and 32% of girls aged 6 years old had class II in Iceland, while 6.2% and 4.8 had class III in boys and girls respectively (Johannsdottir et al, 1997). Thilander et al. found a 21% and 3.7% prevalence

Table 1. Socio-demographics of included children

Demographics		Frequency	Percent
Sex	Male	133	19.39
	Female	553	80.61
Residence	Ho Chi Minh city	385	56.12
	Ha Noi	214	31.20
	Others	87	12.68
Father education level	Less than Secondary	153	22.30
	Secondary	257	37.46
	More than Secondary	276	40.23
Mother education level	Less than Secondary	230	33.53
	Secondary	176	25.66
	More than Secondary	280	40.82

of class II and class III malocclusion in 5-17 years old students (Thilander, et al, 2001). In Lithuanian children, class I, class II and class III occlusions were seen in 68.4%, 27.7% and 2.8% of them (Sidlauskas & Lopatiene 2009). The present study found normal bite in 34.11% of the children, other types of bites include open bite, deep bite, edge to edge bite which were recorded in 47.23%, 15.45%, 3.21% of children respectively. Similar results of Thilander et al, who found 21.6% prevalence of deep bite in 5-17-years-old boys (Thilander, et al, 2001). These findings were different in Lithuanian children where the prevalence of deep bite (>3.0 mm) was 14.5% and the prevalence of open bite was only 3.5% (Sidlauskas & Lopatiene 2009). The transverse relation between dentitions in the studies children were normal in 75.1% of children, while 10.1% and 13.8% of children had anterior and posterior cross bite respectively. In Lithuania, posterior cross bite was found in 8.8% of schoolchildren. (Sidlauskas & Lopatiene 2009). In the present study, the prevalence of diastema was 37.47%, This result is similar to the result of Abumelha et al 40.7% (Abumelha, et al, 2018), while Alzubair and Ghandour found 14.2% prevalence of diastema with 1 mm or more in 12 years children. Thilander et al, reported a prevalence of 13.5% in early mixed dentition that have been decreased with dentition development to be 3.7% in adolescents (Thilander, et al, 2001). Another study among Swedish school children found 5% prevalence of medial diastema. The prevalence of sucking habits among Saudi children was found as high as 48.36% (Farsi & Salama 1997), which explained to some extent the high prevalence of spacing in upper dentition.

Table 2. Distribution of malocclusions among children aged 8-10 years old in HCM region.

	Category	Frequency	Percent
Occlusion	Malocclusion	243	35.42
	Normal occlusion	443	64.58
Type of malocclusions	Class 1	435	63.41
based on molar relation	Class 2 division 1	87	12.68
	Class 2 division 1 with subdivision	40	5.83
	Class 2 division 2	22	3.21
	Class 2 division	14	2.04
	2 with subdivision		
	Class 3	54	7.87
	Class 3 with subdivision	34	4.96
Type of malocclusions	Normal Bite	234	34.11
based on	Open Bite	324	47.23
vertical relation	Deep Bite	106	15.45
	Edge to edge	22	3.21
Type of malocclusions	Normal bite	453	66.03
based on	Anterior crossbite	116	16.91
transverse relation	Posterior crossbite (unilateral)	70	10.20
	Posterior crossbite (bilateral)	31	4.52
	Posterior crossbite (total)	9	1.31
	Over Jet (anterior)	7	1.02

Table 3. Proportion of spacing and teething in children 8-10 years in HCM region

	Category	Frequency	Percent
Teeth crowding	Absent	432	62.97
	Present (Upper)	43	6.27
	Present (Lower)	198	28.86
	Present (Upper and lower)	13	1.90
Diastema	Absent	429	62.54
	Present (Upper)	189	27.55
	Present (Lower)	13	1.90
	Present (Upper and lower)	15	2.19
	Physiological (Upper)	40	5.83
Premature tooth loss	No	563	82.07
	Yes (Deciduous)	34	4.96
	Yes (Permanent)	75	10.93
	Yes (Deciduous and permanent)	14	2.04

The prevalence of tooth crowding in our study was 37.03% while Alzubair and Ghandour found 31.4% prevalence of crowding and Abumelha et al 36.5% (Al-Zubair & Ghandour 2014; Abumelha et al, 2018). Thilander et al, found the crowding as the most common problem affected more than half of the studied 5-17 years aged students (Thilander, et al, 2001). In Lithuanian children, the prevalence of crowding in the upper and lower dentitions was 38.4% and 35.5% respectively. This prevalence was found significantly increased with age of children (Sidlauskas & Lopatiene 2009). This is in accordance with our study that found older children had significantly higher malocclusion prevalence than younger children. This study found more than one third of Vietnam children affected by different types and degrees of malocclusion, mal-alignment and crowding. Significant associations were detected between age of children and prevalence of malocclusion and crowding. These findings will highlight the high treatment needs of orthodontic treatment among Vietnam children.

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Conflict of Interest: The author discloses that there are no conflicts of interest.

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Influence of Maxillary Incisor Variables on Smile Attractiveness: Perception of Saudi Population

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ABSTRACT

To assess the influence of four maxillary anterior teeth-related variables on the smile attractiveness in Saudi population. Two standardized frontal smile photographs with skin color type III (Fitzpatrick scale) & tooth shade BL2 for male and female subjects were used to alter four different variables (lateral incisors length, central incisors angles, length of interproximal contact area and prominence of central incisors). An electronically displayed questionnaire was used to assess the demographic characteristics of subjects along with the satisfaction of their smiles. Each variable had a set of three digitally altered images in which the participant should select the most attractive one. Cross-tabulations and chi-squared tests were used to perform the statistical analyses ($\alpha = 0.05$). 843 subjects were enrolled in the study. The median (IQR) score of importance of smile and level of satisfaction of smile among the study subjects were 9(3) and 7(3) respectively. The comparison of mean rank score of importance of smile, in relation to the age groups, gender and educational level showed statistically significant difference. Mean rank values of level of satisfaction of smile in relation to age groups, gender and educational level were comparable. Most of the Saudi populace preferred 1mm incisal length difference in females and equal incisal length in males, with round incisal angles for both males and females. The ideal interproximal contact area of 50:40:30 proportion was considered as most attractive and almost half of the included Saudi population preferred the prominent central incisor.

KEY WORDS: AESTHETICS; SMILING; MAXILLARY ANTERIOR; SAUDI POPULACE; INCISAL ANG.

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INTRODUCTION

The analysis of the dental esthetic and assessment of various elements that contribute in the production of a pleasing and attractive smile are a major concern for dental practitioners (Niaz et al., 2015). Patients in contemporary dentistry are increasingly concerned with the esthetic of the restoration and their satisfaction is primarily based on the definitive appearance of the dentofacial structures (Exley, 2009). When it comes to dental esthetics, to achieve an excellent result, it is mandatory to provide an interdisciplinary treatment for patients (Rosa and Zachrisson, 2007). Esthetic analysis and planning are of critical importance to achieve patient expectations when providing any esthetic dental treatment. Multiple dental and dento-gingival variables contribute towards achieving an attractive smile (Sepolia et al., 2014 Parrini et al., 2016).

These vital elements can be divided into two different categories. The first one includes tooth-related components including, dental midline, incisal length, tooth dimension, zenith point, axial inclination, interdental contact area, incisal embrasure, symmetry, and balance (Ito et al., 2006). The second category includes soft-tissue related components such as, gingival health, gingival harmony and levels, interdental embrasure, and smile line (Bhuvaneswaran, 2010, Taki et al., 2017). Recently, relationship between perception of smile esthetics and orthodontic treatment in Spanish patients has been reported by Colvee et al. (2018), where patients presented significant differences in the esthetic perception of midline diastema and gummy smile anomalies after they had completed orthodontic treatment. Gender influenced the perception of smile.

Esthetics, whereby women were significantly more critical of midline diastema, black triangle and gingival margin of the upper central incisor than men. The age variable also showed significant differences in the perception of midline diastema and black triangle anomalies. The perception of smile esthetics of some dental anomalies changes as a result of orthodontic treatment. Gender influences the perception of some of the dental anomalies studied, (Colvee et al. 2018). According to literature, features related to maxillary central incisor, forms the foundation to achieve excellent results when designing a smile (Rosenstiel et al., 2000). "The dominance of central incisors" is a term that has been used widely in literature to highlight the significance of central incisors and to refer them as the protagonists of smile (Machado et al., 2013). However, with increasing age patients tend to develop a reverse smile as a result of incisal attrition losing the incisal edge position of maxillary anterior teeth (Saffarpour et al., 2016). Anderson et al., (2005) in a study concluded that the shape of the canine was less important than the shape of central and lateral incisors in the esthetics of anterior teeth. In addition, it is reported that round shaped incisors (i.e., round outline form of the tooth) were more esthetically pleasing than their square counterpart. Furthermore, an enlarged gingival embrasure called "black triangle" cervical to the contact point of incisal teeth is of great significance, affecting the perception of patients on smile (Sudhakar and Vishwanath, 2014, Al Taki et al 2017 Geevarghese et al., 2019). The criteria by which individuals perceive and define beauty differs based on multiple factors including social characteristics and profession of an individual (Al Taki et al., 2017).

A study by Labban et al., (2017) previously investigated the influence of gender and skin color on the preference of tooth shade in Saudi population. However, there is limited evidence on the effect of maxillary incisor variables on the perception of different smiles in Saudi population. In the present study, it is hypothesized that there is no difference in the perception of different smiles when changing the maxillary incisors related variables. Therefore, the aim was to assess the Saudi population perception of different smiles by altering the variables of maxillary lateral incisors vertical position, shape of the mesial and distal angles, interproximal contacts, and the prominence of central incisors (i.e., wider tooth width resulting in greater percentage of anterior width).

MATERIALS AND METHODS

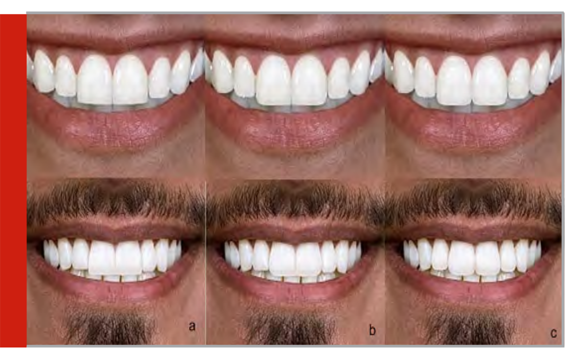
Preparation of photographs: The study protocol was approved by the Institutional Review Board (IRB) & the College of Dentistry Research Centre (CDRC). Twenty-Two digital photographs for two adult subjects (male & female) were used in the present study. Photographs showed the frontal smile alone, excluding other facial structures to avoid any distracting variables. Skin color type III (Fitzpatrick scale) & tooth shade BL2 (A-D shade guide 2016 Ivoclar Vivadent Inc., USA) was used

based on the results of our previous study (Labban et al., 2017). The teeth shape parameters in the photographs were digitally altered by Adobe Photoshop software (Adobe Systems CS6, Ireland Ltd).The photographs were grouped into four sets for both male and female, each representing an altered maxillary incisor related variable as follows: (1) Crown length of the lateral incisors, (2) The shape of the maxillary central Incisor angles, (3) Length of interproximal contact area, (4) Prominence of central incisors.

Crown length of the lateral incisors: The Incisal edge of the lateral incisor was altered by 1 mm increments to produce three images: 0 mm, 1

mm, 2 mm shorter than the adjacent central incisor (Fig. 1).

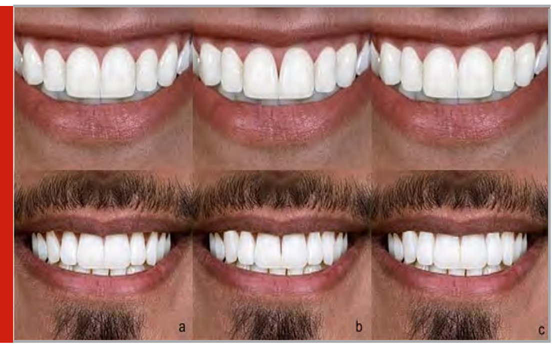
The shape of the maxillary central Incisor angles: Mesial and distal angle was altered to produce



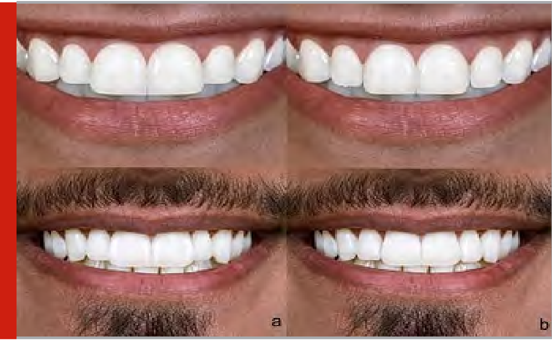
three images: square, square-round, and round (Fig. 2).

Length of interproximal contact area

The length of the maxillary centrals was measured using a tool in Photoshop software, and divided into a nominal 100 units. This calibration was used to modify the interproximal contacts area between the maxillary anterior teeth with different ratios. The three pictures investigated were as follow: The 'Ideal' image giving a ratio of 50:40:30. The "Equal" image interproximal contact areas as arranged in a 50:50:50 ratio. The "Reversed" image demonstrates the interproximal contact areas as arranged in a 30:40:50 ratio. (Fig. 3).



Prominence of central incisors "Altered Smile": Two maxillary central incisor images were manipulated in both width and length. As for the length, the central incisors were elongated by 0.5 mm in both male and female. As for the width, the central incisors were manipulated in a way to give an illusion that was more prominent than the adjacent lateral incisors. (Fig. 4).



Questionnaire distribution and data collection: Consent was obtained from each participant prior to their enrolment in the survey. The subjects were randomly selected from shopping malls and public areas. Exclusion criteria included dentists and children. The questionnaire was electronically displayed to the subjects on a tablet device (Apple iPad pro tablet, Apple Inc.). The questionnaire included item to assess demographic characteristics such as age, nationality, gender,

educations level, occupation, and income. The subjects were asked to report level of satisfaction of their smiles on a scale from 0 to 10 (0, not satisfied and 10, very satisfied). Applicants were also asked to indicate the importance of a smile using the same scale (0, not important and 10, very important). Sets of male and female pictures were displayed to the subjects and they were asked to select the most esthetically pleasing smile regarding gender and teeth shape. Therefore, each participant was asked to prefer a total of 8 images from the total of 22 different images showed and asked to describe the characteristics from these images. Statistical analysis: Data were analyzed using SPSS (version 21, SPSS Inc., Chicago, IBM, USA) statistical software. Descriptive statistics (median, inter quartile range, frequencies, and percentages) were used to describe the skewed quantitative and categorical variables. Mann-Whitney U-test and Kruskal Wallis test were used to compare the mean rank values of importance of smile and satisfaction level in relation to the categorical variables of two and more than two categories. Pearson's Chi-square test was used to observe the association between the categorical variables. A significance level of 0.05 was used to report the statistical significance of results.

Table 1. Distribution of socio-demographic characteristics of study participant (n=843)

Characteristics	No (%)
Age groups (in years)	
20 to 30	488(57.9)
31 to 40	222(26.3)
41 to 50	113(13.4)
Above 50	20(2.4)
Gender	
Male	163(19.3)
Female	680(80.7)
Educational level	
School level	111(13.2)
Bachelor level	704(83.5)
Higher level	28(3.3)
Occupation	
Unemployed	679(80.5)
Employed	164(19.5)
Monthly income (in Saudi Riyals)	
<3000	537(63.7)
3000 to 7000	91(10.8)
7001 to 15000	69(8.2)
>15000	146(17.3)

RESULTS AND DISCUSSION

Out of 843 subjects in the present study, 488 (57.9%) were in age range between 20 to 30 years. Majority of the respondents (80.7%) were females.

Table 2. Subject preference of esthetically pleasing smile descriptive statistics of importance, level of satisfaction of smile

Characteristics	No (%)
Picture 1 (female)	
Equal incisal length	231(27.4)
2 mm shorter incisal length	302(35.8)
1 mm shorter incisal length	310(36.8)
Picture 1 (male)	
Equal incisal length	358(42.5)
2 mm shorter incisal length	299(35.5)
1 mm shorter incisal length	186(22.1)
Picture 2 (female)	
Incisal angle Round-Round	449(53.3)
Incisal angle Square-Square	333(39.5)
Incisal angle Square-Round	61(7.2)
Picture 2 (male)	
Incisal angle Round-Round	420(49.8)
Incisal angle Square-Square	236(28.0)
Incisal angle Square-Round	187(22.2)
Picture 3(female)	
Interproximal contacts 50,40,30	485(57.5)
Interproximal contacts 30,40,50	263(31.2)
Interproximal contacts 50,50,50	95(11.3)
Picture 3 (male)	
Interproximal contacts 50,40,30	495(58.7)
Interproximal contacts 30,40,50	327(38.8)
Interproximal contacts 50,50,50	21(2.5)
Picture 4 (female)	
Altered smile	414(49.1)
Normal smile	429(50.9)
Picture 4 (male)	
Altered smile	429(50.9)
Normal smile	414(49.1)
Importance of smile (Median & IQR)	9(3)
Level of satisfaction (Median & IQR)	7(3)

Bachelor level of education was found in 704 (83.5%) subjects. 679 (80.5%) respondents were unemployed and the monthly income of >15000 SR was found in only 17.3% subjects (Table 1). Subjects' preference for these characteristics towards four male and female pictures which displayed different variables (incisal length, incisal angel, interproximal contacts areas and prominence of centrals) are given in table 2. The median (inter quartile range) score of importance of smile and level of satisfaction of smile among the study subjects was 9(3) and 7(3) respectively. (Table 2). The comparison of mean ranks of scores of importance of smile, in relation to the age group, gender, educational level expressed statistically significant difference. Whereas, level of satisfaction of smile was statistically comparable to different age groups, gender, and educational level (Table 3). When compared different age groups, to study variables of esthetically pleasing smile, high percentage of subjects in age group, 20 to 30 years preferred equal incisal length (47.3%, 51.8%) and 2mm shorter lateral incisal lengths (73.4%, 25.8%). However, those in age range of 41 to 50 and above favored 1 mm shorter lateral incisal length. Similar responses were observed towards the female and male pictures with three categories of incisal angle across the four age groups, indicating significant difference. The responses toward female and male pictures of two categories of prominent central incisors across the four age groups showed significant difference. The highest percentage among male and female (81.1% and 84.4%) responders in age range of 20 to 30 years, preferred altered smile. However, 91.9% & 92.3% of subjects in age range of 31-40 years preferred non-altered smile respectively. All subjects in age group of 41-50 and above 50 years preferred normal smile among both the female and male images respectively. Considering

Table 3. Comparison of mean ranks of (i) importance of smile (ii) satisfaction level of smile scores in relation to socio-demographic characteristics of study subjects

	Importance of smile			Level of satisfaction of smile		
	Median (IQR)	Mean Ranks	p-value*	Median (IQR)	Mean Ranks	p-value*
Age groups (in years)	8(3)	281.75	<0.0001	7(3)	428.85	0.437
20 to 30	10(0)	601.86		7(3)	400.59	
31 to 40	10(0)	651.39		7(3)	437.62	
41 to 50	10(1)	559.58		5.5(5)	404.23	
Above 50 Gender	10(2)	505.0	<0.0001	7(3)	424.52	0.882
Male	9(3)	402.11		7(3)	421.39	
Female Educational level	10(0)	602.28	<0.0001	7(3)	433.70	0.652
School level	8(3)	387.50		7(3)	421.55	
Bachelor's degree	10(1)	574.64		6(2)	386.93	
Higher degree Occupation Unemployed	9(3)	393.10	<0.0001	7(3)	424.08	0.610
Employed	10(1)	545.79		6(3)	413.37	
Monthly income (in Saudi Riyals) <3000	8(2)	317.08	<0.0001	7(3)	419.57	0.170
3000 to 7000	10(0)	611.63		6(3)	393.53	
7001 to 15000	10(2)	518.22		6(3)	403.04	
>15000	10(0)	644.25		7(3)	457.65	

gender, higher proportion of males (52.8%) preferred 1mm shorter lateral incisor length of female picture, while (43.6%) of male preferred 1mm shorter lateral incisor length of male picture. Similarly, in female and male pictures of interproximal contacts, 42.9% male preferred interproximal contact ratio of 50:40:30 in female picture. Whereas, 61% of female preferred interproximal contacts 50:40:30 in female picture. This comparison was found to be statistically significant. Furthermore, statistically significant association was found between the preferences of two categories of prominent centrals in the male picture, in which 43.6% & 56.4% of male had preferred altered and non-altered smile respectively. When education level of subjects was compared to different study variables.

It was observed that those who had bachelor's degree had skewed views regarding the length and shape of lateral incisors both for male and female smiles. Whereas, subjects with a higher degree (100%) preferred 1 mm shorter lateral incisor length both in male and female images. Among the respondents who studied till secondary school, a majority preferred the picture of interproximal contacts 30:40:50 in female smiles and interproximal contacts 50:50:50 in male smiles. Whereas, most of the respondents with bachelor's degree, opted for 50:40:30 both in male and female picture. Majority of subjects (95.5%) having education level till school preferred non- altered smile both for males and females. In addition, majority of subjects having bachelors level education, chose rabbit smile for both genders. All subjects with higher level of education preferred non-altered smile in both female and male smile.

Esthetic dentistry is understood as a general approach to enhance the standards of beauty alongside the optimal health and function of the oral cavity. The present study was based on the hypothesis that there is no significant difference in the perception of different smiles when changing the maxillary incisors related variables. Interestingly, the hypothesis was rejected as changing maxillary incisors variables altered the perception of different smiles. This type of survey was distinctive, as to our knowledge this is the first study of its kind to be conducted in Saudi Arabia.An ideal step between the central and lateral maxillary incisors range between 1 to 1.5 mm for females and 0.5 to 1 mm for males. This indicates that convex smile arcs are more esthetically pleasing for females and convex to plane arcs are more esthetically pleasing on males (Machado et al., 2013). The results of this current study revealed that a majority (52%) of Saudi male subjects preferred 1 mm step for females. Almost 38% of the Saudi female subjects preferred 2 mm step for females and 45% preferred equal incisal length for males. The overall population's perception regarding the difference in the incisal length between the central and lateral incisor show that most of the population prefer 1mm difference for females and equal incisal length for males. A similar study by Taki et al., (2017) showed results which were in contrast to the present study. The results by Taki et al, (2017) displayed no significant difference according to gender in the preferred length of lateral incisor. An incisal difference of 0.5 mm was preferred by general populace both in males and females in their study (Taki et al., 2017).

When the opinions of all subjects were viewed collectively, regarding their preference of the shape of the central and lateral incisors, most of the subjects chose round angles for both males and females. In a similar study Anderson et al., (2005) aimed to evaluate the contribution of the maxillary incisors' shape on smile esthetics. The study concluded that the round shape of the mesial and distal angles was preferred on female subjects by general practitioners and orthodontists while for common population, there was no preference. In contrast, the square to square- round angles were favored esthetically on males by all orthodontists, general practitioners and lay-people. A disparity regarding perception in shape of lateral and central incisor among females exists within the results of present study and the study by (Anderson et al., 2005).

When considering the effect of the interproximal contact area between the central incisor, lateral incisor and canine, a previous study by Foulger et al., (2010) concluded that 'equal' (50:50:50) interproximal contact area arrangement is deemed as most attractive.16 This is in contrast to the ideal 50:40:30 ratio proposed by Sudhakar and Vishwanath, (2014) Conversely, an interproximal contact area arrangement resulting in the presence of a 'black triangle' was deemed least attractive. Comparing the results of study by Foulger et al., (2010) to the present study that focused on the Saudi population perception, it was noticed that the majority of the population preferred the ideal 50:40:30 proportion for both

males and females. However, it was also observed that subjects with higher degree (educational level) and subjects above the age of 40 preferred the equal interproximal contact with the ratio of 50:50:50 for females and the reversed interproximal contact arrangement with the ratio of 30:40:50 for males.

A recent emerging tendency in which individuals prefer having a prominent maxillary central incisor tooth, is now considered as a new criterion in dental esthetics. It is noticed that large number of patients attending dental clinics seek such profile. This variable has not been mentioned nor studied in the literature before, specifically in Saudi population. Furthermore, it has not been evaluated as previous mentioned criteria on how it affects and determine one's smile esthetics' and attractiveness. The results of the present study revealed that almost half of the Saudi population preferred the "altered smile" on both male and female subjects, which indicates high acceptance level of this trend as new criteria of achieving optimal dental esthetics. It was also noted that the male and female subjects who preferred the "altered smile" look, were mostly in the age group of 20-30 with the percentage of 81% and 84% in male and female subjects respectively.

While most of the participants who were in the age group of 31- 40 and above, preferred the normal central incisors profile. In summary, our results demonstrate that whilst there is broad agreement amongst the groups in terms of what constitutes optimal esthetics, differences exist and unless communication is effective, individual pre-conceptions may compromise the patient's acceptance of the result. In addition, based on the findings and limitations the authors recommend that a comparative study with people of different cultural backgrounds can contribute immensely in understanding variables that may influence an ideal smile. Moreover, since majority of this sample was in the twenty to thirty-year age range, over 80 percent were female, and two-thirds have a monthly income in the lowest income category these demographics might have an influence on the generalization of results. Hence, future studies with increase sample size and diverse demographic characteristics are recommended.

CONCLUSION

It is concluded that variations in the length, shape and prominence of maxillary anterior teeth, particularly the central and lateral incisors, can influence people's perception of the overall smile attractiveness. Gender, age group, educational background can influence one's judgment and opinion regarding the standards of beauty and attractiveness.

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Orthodontic Pain Induced Sensitization of Trigeminonociceptive System of Patients Undergoing Fixed Orthodontic Treatment

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ABSTRACT

Pain is a complex sensory experience and often accompanies orthodontic conditions and interferes with its management. Orthodontic pain induced by application of orthodontic appliances and traction forces on mal-occluded teeth, is typically transient (peaks in 24 hours and subsides within a week), alters sensory perception and normal motor activities such as chewing and talking. The present study was aimed to record thermal sensitivity and motor activity of masseter muscle in patients undergoing fixed orthodontic therapy at 3 time points i.e. before start of treatment (Day 0), at 24 hours (Day 2) and a after a week (Day 7). Pain rating by visual analog scale, Quantitative sensory testing (thermal detection, pain threshold and tolerance threshold for both cold and hot stimuli) and surface electromyography (amplitude and time to fatigue) were recorded for masseter muscle. D'Agostino-Pearson and Friedman's test were applied for determining normality and for comparisons respectively. Twenty eight patients (17 males, 11 females) mean age (17.88 ± 3.92 years) were recruited from Orthodontics and maxillofacial deformity department. We found significant changes in cold pain perception and cold pain tolerance at Day 2, when compared to Day 0 and Day 7. At Day 2 amplitude of maximum voluntary contraction and time to fatigue were significantly lower than Day 0. All other parameters such as thermal thresholds were comparable at all the time points. The findings suggest that orthodontic procedures causes transient sensitization of the trigeminal nociceptive system. Quantitative assessment of somatosensory and motor function may help in better understanding of time kinetics and mechanism of orthodontic pain in order to plan and modify orthodontic treatment based on patients thresholds and responses.

KEY WORDS: *ELECTROMYOGRAPHY, ORTHODONTIC PAIN, PERIPHERAL SENSITIZATION, QUANTITATIVE SENSORY TESTING.*

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INTRODUCTION

Pain is an unpleasant somatic and emotional experience associated with actual or potential tissue damage. Pain can also be acute or chronic in nature, it includes sensations evoked by, and reactions to noxious stimuli. Pain is a complex experience and often accompanies orthodontic procedures. Orthodontic pain is the most common complaint resulting from application of orthodontic forces for correction of malocclusion and is a major cause of reduced patient compliance. Orthodontic forces cause movement of mal-aligned teeth that results in an inflammatory process in the periodontal ligament (PDL). It has been correlated with the release and presence of various substances, such as substance P histamine, enkephalin, dopamine, serotonin, glycine, glutamate gamma-amino butyric acid, PGEs, leukotriens, and cytokines. Malocclusion is not only esthetically but also functionally compromising.

Orthodontic grip forces induce orthodontic pain which not only alters sensory perception (feelings of pressure, tension and soreness of the teeth) but also makes normal muscular activities difficult for the patients, (Hosseinzadeh et al., 2016, Topolski et al., 2018). In a previous study to check sensitization of teeth, the various discomforts experienced by patients after appliance placement are described by them as feelings of pressure, tension, soreness of the teeth, and pain (Ngan et al., 1989). In another study pain escalated 2-4 hours post application of appliances and traction forces, reached peak levels in 24 hours and subsides within a week (Bergius et al., 2000). Despite some studies there is a paucity of research that has systematically explored the changes in the nociception, somatosensory and muscular activity at extra-oral sites during fixed orthodontic treatment. The presence of widespread sensitization is still unclear in the surrounding muscles. More over some studies suggest changes in motor activity which is inferred as a protective reflex following traction.

(Scheurer et al. 1996, Michelotti et al., 1999, Bondemark et al., 2004). The study of both somatosensory and motor function helps in understanding the underlying pathophysiological mechanisms of orthodontic pain. Quantitative sensory testing (QST) is a non-invasive psychophysical testing method in which different modalities (thermal, mechanical, electrical, and chemical) are applied to different tissues (skin, mucosa, muscles and joints) and perception threshold are noted. Electromyography (EMG) is a common electrodiagnostic technique used by orthodontists for evaluating and recording the electrical activity of the muscles. An electromyographic EMG study performed to evaluate the motor and sensory changes associated with separator placement, showed a decrease in motor output as well as pressure pain threshold in muscles of mastication. Michelotti et al.,1999. In the present study we explored sensory and motor aspects of masseter function in patients undergoing fixed orthodontic therapy.

MATERIAL AND METHODS

Patient characteristics: Twenty patients (11 males and 9 females) of different classes of dental malocclusion (Angle's Class I, II and III) were recruited from the Orthodontics and dentofacial deformities department of Centre for Dental Education and Research, All India Institute of Medical Sciences (AIIMS). The mean age of subjects was 17.88 ± 3.92 years. All tests were performed at Pain Research and TMS Laboratory, in Department of Physiology at AIIMS, New Delhi. Consent form for procedures, which were further approved by the Institute's ethical committee (IECPG-248/30.03.2016, RT-17/27.04.2016) was taken from the subjects and thereafter the tests were performed. Non-surgical cases with dental malocclusion aged 13-25 years (for <18 years of patients, guardian was there) with normal growth pattern (FMA-25°-27°) and normal masseter muscle activity were recruited. Patients with current orofacial inflammatory conditions, history of dental or orthodontic treatment 3 months prior to the study, taking medications and any neurological or metabolic disorders were excluded from the study. Quantitative Sensory Testing and Electromyography tests were done on the area overlying masseter muscle at 3 time points once before the start of fixed orthodontic therapy (Baseline, Day 0), then 24 hours after application of appliances and traction forces

(Day 2) and finally; after a week (Day 7). At first visit history was taken (both dental and medical), thermal thresholds and motor activity of masseter were recorded. At second visit, (Day 2) patient was asked to report pain experience and the sensory and motor tests were repeated. At third visit, (Day 7) re-evaluation of pain, sensory and motor activity was done. The study was conducted in a silent, isolated room with an ambient temperature between 21 ^{0}C and 25°C. During the QST, participants were instructed to close their eyes and the tests were performed according to the standard protocol. The QST included the following variables: Thermal detection thresholds (Cold detection threshold-CDT, Warm detection threshold-WDT), pain and pain tolerance thresholds (Cold Pain threshold-CPT, Cold pain tolerance threshold-CPTT; Hot Pain threshold-, HPT, Hot Pain Tolerance threshold-HPTT).

The thermal thresholds were recorded using a thermode (30 mm X 30 mm) probe over left masseter muscle and first web space of left hand (as a reference site). (Fig 1) The CDT and the WDT were always measured first, followed by the CPT, HPT, CPTT and HPTT. The baseline temperature was set at 32°C, the temperature increased or decreased, at a rate of 1°C/s, to the lower limit of 0°C or to the upper limit of 50°C, respectively. Subjects were instructed to press a button as soon as they perceived the respective thermal sensation of cold, heat, cold pain, and heat pain and when they could no longer tolerate the cold or hot stimulus. BioPac, Student laboratory system, BSL Pro (V 3.6.7 model), with pre-gelled. disposable Ag-AgCI cup electrodes (Biopac, UK) were utilized to record surface. EMG signal from masseter muscle, connected to the MP30 unit through smart. sensors. Recordings were done at rest and at maximum voluntary contraction. (MVC) and the results were expressed as amplitude and time to fatigue.

Figure 1. Time to fatigue and amplitude of Maximum voluntary contraction of Masseter is compared at the three time points

Data Extraction: Data acquisition was done in BioPac, student laboratory system, BSL Pro and Neurosensory analyser TSA-II and extraction was done in MS Office Microsoft Excel 2013.

Statistical analysis: Descriptive statistics were used to summarize all measurements. The mean and standard deviation values of pain rating (VAS), motor activity (amplitude and time to fatigue at maximum voluntary contraction) were calculated and compared using one way ANOVA. Data was found to be non-parametric for QST data (thermal thresholds- CDT, WDT, CPT, HPT, CPTT, HPTT) at masseter and at hand but parametric for EMG data. Friedman's test with repeated measures was used to assess the time effects and represented as median with range.

RESULTS AND DISCUSSION

The results of 20 patients are represented. Pain, sensory and motor evaluations were done at all the 3 time points (Day0, Day 2 and Day 7 respectively).

VAS rating for Pain: At baseline the patients were pain free (VAS=0), the pain intensity reached a peak at 24 h (VAS = 5.3, range: 1.9–8.9) and thereafter there was a gradual reduction in the pain intensity by Day 7 (VAS = 1.5; range: 0.5–2.6).

QST findings: We found significant changes in cold detection thresholds (CDT), cold pain thresholds (CPT) and cold pain tolerance thresholds (CPTT) at day 2 at masseter but at day 7 they were comparable to their respective baseline (day 0) values. We found cold detection (p= 0.004) and cold pain tolerance thresholds (p= 0.0001) to be significantly higher when patient had maximum pain i.e at day 2 compared to baseline when patient was pain free. Cold pain threshold decreased significantly at day 2 (p=0.003). Thermal detection, pain and pain tolerance thresholds for the hot stimuli were all comparable at the 3 time points.

EMG findings: The motor activity changes of masseter at maximum voluntary contraction when recorded at 3 time points. Amplitude (at rest) at baseline was 1.22 ± 0.76 mV and on Day 2 it was significantly reduced to 0.53 ± 0.39 mV, on Day 7 the EMG activity at rest and also at MVC were comparable to baseline values.

Sensory motor correlations: The associations were found between thermal sensory parameters and the motor activity of Masseter muscle at the 3 time points i.e. Day 0, 2 and 7. We are presenting correlations and r values. The significant differences were found between the males and females of their motor activities of Masseter muscle and no significant differences were found in the duration of Masseter muscles activity at MVC at all the three time points .The amplitudes of MVC at Day 2 and 7 were significant (p = 0.051). Thermal sensory parameters showed variable results of CDT values for both at baseline and they were comparable but at day 2 and at day 7 significant differences were found (p =0.029, and p =0.018 respectively). WDT values showed significant differences at baseline at day 7 (p =0.042 and p =0.048 respectively). CPT values were significantly different only on day 2 (p =0.046).

HPT values were comparable for both subjects. Females had lower thresholds and lowered pain tolerances at all the times they differed significantly from their male counterparts. Orthodontic tooth movement is known to cause inflammatory reactions in the periodontium and dental pulp, which release various biochemical mediators such as substance P, histamine, enkephalin, dopamine and serotonin which elicit hyperalgaesia (Yamasaki et al., 1984; Walker et al.,1987; Davidovitch et al., 1988; Nicolay et al., 1990; Davidovitch, 1991; Saito et al., 1991b; Grieve et al. , 1994; Alhashimi et al. , 2001, Gupta et al., 2014, Al Sayed et al., 2017). Processing of complex information arising from mechanical force application induces recruitment of neurons, which act by the way of chemical mediators as modulators of the effector response to the stimulus (Vandevska-Radunovic, 1999). Apart from the classic constituents mentioned above, peripheral nerve fibres also participate in the inflammatory process associated with tooth movement (neurogenic inflammation). This involves release of neuropeptides after antidromic stimulation of afferent nerve endings and initiation of an inflammatory reaction. These neuropeptides released are known to elicit a painful response (Vandevska-Radunovic, 1999). Kato et al. (1996) examined the distribution of nerve fibres containing neurofilament protein.

(NFP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and neuropeptides Y (NPY) in the PDL of the rat first molar after mechanical force application. Our study is also supported by Jones 1984 that pain is experienced by the majority of patients 4 hours after archwire placement, which further peaks at 24 hour and then It gradually declines. The mechanism of pain which is caused by orthodontic forces are not fully understood till know. Kamogashira et al, 1988 and Marklund et al, 1994 reported that pain perceived has been related to levels of prostaglandin and substance P in the periodontal ligament and which may be probably related to inflammatory response. It may also injure periodontal nerve terminals which was reported by Loescher et al, 1993.

Our study is in accordance with previous findings of Ngan et al, 1989. The study suggested that the application of orthodontic separators over 24 hour period induces pain in the teeth and surrounding musculature. We studied thermal sensitivity through quantitative sensory testing because for the evaluation of somatosensory function the QST

parameters are known for providing the small fiber function and therefore offer a diagnostic sensitivity of 67% -70% for small fiber neuropathy (Tobin 1999, Hansson 2007). CDT is considered to represent the response mediated by small myelinated Aδ nerve-fiber conduction, as in relative terms, A delta fibres carry messages at the speed of a messenger on a bicycle. Warm Detection Threshold represent unmyelinated C nerve-fiber conduction, and the responses for noxious Cold and Hot pain may be conducted by both Aδ and C nerve fibers (Yarnitsky 1991, Ziegler 1999). The patients had decreased sensitivity to cold stimuli and increased tolerance to cold pain suggesting sensitization at the level of Aδ fibres mainly. Since no significant differences were found in the thermal thresholds for warm stimuli, this may be attributed to the fact that the unlike the cold receptors, warm receptors act more like simple thermometers; their firing rates rise monotonically with increasing skin temperature up to the threshold of pain and then saturate at higher temperatures. Thus they cannot play a role in signaling heat pain. They are much less sensitive to rapid changes in skin temperature than are cold receptors. C fibres that carry hot pain sensations are activated at higher temperatures applied for longer duration.

The EMG study showed significant decrease in massetric muscle activity during maximal clenching and chewing. Our reports are in agreement with Goldreich et al, 1994 who also reported that there is reduction in activity of masseter after orthodontic archwire adjustment. In our study there is decrease in motor activity of masseter because of orthodontic pain and masseter muscle was tender and they had difficulty in speaking, chewing and swallowing. The basal tone of masseter was not affected by the pain. Trigeminal nerve provides tactile, proprioceptive, and nociceptive afferents to the face and mouth, and motor function activates the muscles of mastication (Masseter, Medial and Lateral Pterygoid, Temporalis). Such innervation is responsible for the elaborate peripheral feedback and feed-forward information that is essential for the coordination of masticatory muscles in a predictive manner based on learned relationships between patterns of receptor signals and appropriate efferent signals (Trulsson, 2007). Any alteration in the orofacial environment, either from changes in the consistency of the diet or dental manipulations may affect the exteroceptive, proprioceptive and perhaps also the nociceptive inputs into the CNS and thereby may alter the patterns of jaw and tongue movements during mastication (Murray et al, 2004; Sessle, 2006). Lund et al 1994, explained changes that motor programs control the premotor nociceptive interneuron to agonist and antagonist motor neurons in a reciprocal way. To account for the lowering of agonist output in the presence of pain, the motor command includes excitation (or facilitation) of the inhibitory group and the inhibition (or disfacilitation) of the excitatory group supplying agonist motor neurons. In the presence of pain, the agonist muscles' activity is lowered because of the inhibitory pathway being stimulated and the excitatory pathway being inhibited. In case of orthodontic therapy, application and activation of arch wires would induce the constant forces on the fibers of the periodontal ligament during the course and hence the agonist muscle activity would tend to decrease. At baseline we found absolutely no pain and hypo/hyper activity in masseter (inclusion criteria).

We recorded the muscle activity at rest and at maximum voluntary contraction. The above results suggests that pain arising due to application of constant forces on the fibers of the periodontal ligament during orthodontic treatment, agonist muscle i.e. masseter activity decreases because the inter neurons receive convergent excitatory inputs from different tissues. Similar results were reported by Michelloti et al, 1999, who found significant differences in activity of motor activity before and after the start of orthodontic therapy. Since orthodontic pain is subjective in nature and gender differences are found. Ngan et al., 1989; Erdinç et al., 2004 in their respective studies found adolescent females undergoing orthodontic treatment had lower pain thresholds and pain tolerances compared to age matched Thermal detection, pain and pain tolerance thresholds for the hot stimuli were all comparable at the three time points.

CONCLUSION

There is marked peripheral sensitization at the level of nociceptors at a distant site other than oral cavity i.e. at masseter from the orthodontic therapy, also not only thermal hyposensitivity to cold stimuli was seen, also patient's tolerance to cold pain stimuli increased and motor activity was adversely affected. The study of basal thermal sensitivity and motor activity of masseter muscle before the start of orthodontic treatment will benefit orthodontists in planning and modifying the treatment for patients. Thus QST and EMG may become useful tools during orthodontic therapy for better patient compliance.

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Accumulation of Cytotoxic Zinc and its Detoxification by *Brassica napus*, Mediated by Endophytic Bacteria

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ABSTRACT

Brassica napus is a heavy metal hyperaccumulator plant which can tolerate high levels of heavy metals as compared to other plants. In the present study, *B. napus* was exposed to 20 and 50 mg/kg of Zn and 0 mg/kg of Zn treated plants, which were used as controls. The results showed that high Zn concentration reduced plant shoots length, roots length, chlorophyll contents and biomass in a dose-dependent manner as compared to controls. However, inoculation of bacterial isolated, *Serratia sp*. IU01 significantly improved ($p < 0.05$) all plant growth attributes as compared to uninoculated plants. The estimation of Zn accumulation through inductively coupled plasma mass spectrometry (ICPMS) in shoot and roots showed that a higher concentration of Zn was accumulated in roots as compared to shoot and inoculation significantly improved ($p < 0.05$) the Zn accumulation in roots as compared to uninoculated plants..

KEY WORDS: ZINC TOXICITY, HEAVY METAL, IMMOBILIZATION, BRASSICA NAPUS, GROWTH ATTRIBUTES.

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INTRODUCTION

Endophytes are plants symbiotic microbes (bacteria and fungi), provide physiological support to the plants. They help plants in development and growth promotion (Jeger and Spence, 2001). The main benefit of the endophytic bacteria is assumed to be the stress resistant and make the plants stress resistant as well, (Sturz et al., 2000). The heavy metal stressor are very common in present age (Jeger and Spence, 2001; Bharti et al., 2016). From soil to plant, transport of heavy metals depends on total amount of heavy metals, in soil and rate of transfer of heavy element from soil to plant roots (Buendía-González et al., 2010). Numerous studies have described that endophytic bacteria can apply for agricultural purposes to enhance the phytoremediation role of the heavy metal accumulator plants (Sturz et al., 2000). In addition endophytes perform nutrients mobilization such as nitrogen production and phosphorus solubilization (Sharma et al., 2013), providing plant hormone such as auxin and gibberellin (Hardoim et al., 2008) and protect the diseases caused by soil-borne pathogens and hence plants are enabled to tolerate that heavy metal toxicity and go for phytoremediation, (Bahadir et al., 2007; Ullah et al., 2019).

The heavy metal such as Zn has been a major threat to animals, plants, and human life due to their toxicity in living organisms. Contamination of soil with heavy metals enhances plant uptake leading to accumulation in various parts of the plants (Berg and Hallmann, 2006). Larger agricultural soil has been contaminated with heavy metals due to mining activities, industrial discharges and the application of agrochemicals and lime products (Bahadir et al., 2007). Severe heavy metal contamination in the soil may cause a variety of problems, including low crop yield and toxicity in plants, animals, and humans. A trace amount of few heavy metals such as Cu and Zn are essential for plant growth and natural development because they are used as co-factors for many enzymes (Ullah et al., 2015). However, high concentrations of both essential and unnecessary heavy metals in the soil can lead to toxic symptoms and growth inhibition in most of the plants (Dahmani-Muller et al., 2000). At the cellular level, excessive amounts of toxic heavy metal ions stimulate many stress responses and damage various cell components such as cell membranes, proteins and nucleic acid (Fidalgo et al., 2011; Chauvin et al., 2017). Bacterial mediated phytoremediation have been used to detoxify the heavy metal such as zinc (Zn) by immobilization and turn them harmless to the soil, water or air (Buendía-González et al., 2010).

Contaminants such as minerals, pesticides, solvents, explosives, crude oil, and derivatives have been detoxified by bacterial mediated phytoremediation of plant such as Brassica napus (Fidalgo et al., 2011). Phytoremediation technique is a method of treatment that takes advantage of the ability of hyper-accumulator plants to accumulate heavy metals and toxic compounds

Table 1. Plant growth characteristics of Brassica napus under various concentrations of Zn

Zn Treatment (mg/kg)		Shoot length (cm)	Root Length (cm)	Chlorophyll contents (SPAD)	Fresh biomass	Dry biomass
0	Uninoculated	12.46 ± 1.22[a]	12.33 ± 2.81[a]	22.11 ± 2.23[a]	60.54 ± 3.22[a]	21.62 ± 1.35[a]
	IU01	11.96 ± 1.52[a]	13.35 ± 2.53[a]	23.53 ± 3.13[a]	62. 43 ± 2.46[a]	23.41 ± 2.33[a]
10	Uninoculated	8.40 ± 1.38[b]	08.00 ± 1.43[b]	15.21 ± 1.67[b]	40.33 ± 1.53[b]	12.29 ± 0.66[b]
	IU01	10.96 ± 1.52[a]	11.00 ± 1.36[a]	21.74 ± 2.62[a]	57. 88 ± 2.76[a]	20.56 ± 2.45[a]
50	Uninoculated	5.38 ± 0.57[c]	6.25 ± 0.56[c]	12.56 ± 2.67[c]	15. 83 ± 1.45[c]	09.25 ± 0.56[c]
	IU01	9.84 ± 1.71[a]	10.71 ± 1.58[a]	20.56 ± 2.72[a]	52. 37 ± 2.41[d]	18.86 ± 2.75[d]

Mean ± SD values are presented in each column different letters represent the significant difference ($p < 0.05$) as analyzed by Duncan's multiple range test.

from the environment and metabolize them in their tissues (Garbisu and Alkorta, 2001). *B. napus* has been used in studies of the effects of heavy metals such as cadmium hyper-accumulator (Ullah et al., 2013; Xia et al., 2016).

MATERIALS AND METHODS

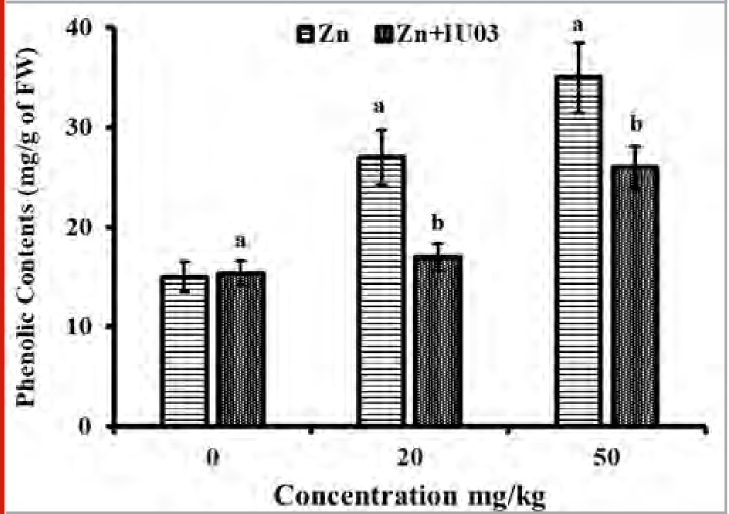
Plant growth, Zn contamination and bacterial inoculation *Brassica napus:* plants were grown in pots using (Wei et al., 2009) commercial soil. The plants were put in a growth chamber at 25 ± 2 °C. Two different Zn concentrations: 20 mg/kg and 50 mg/kg of soil were used as treatments and 0 mg/kg was (without Zn) was used as control. The experiment lasted for 4 weeks. Three replications were used and ten plants per replication were used in the experiment. Previously isolated bacterial strain *Serratia sp.* IU01 (Ullah et al., 2019) was

Table 2. Fresh and dry biomass of B. napus after treatment with different concentrations of Zn (mg/kg of soil).

Zn Treatment (mg/kg)	Treatment	Zn concentration mg/kg of Plant DW*	
		Shoot	Root
0	Uninoculated	ND*	ND
	IU01	ND	ND
10	Uninoculated	250.86 ± 5.95[a]	453.54 ± 5.57[a]
IU01		121.37 ± 3.54[b]	216.28 ± 7.25[b]
50	Uninoculated	327.28 ± 6.53[c]	752.34 ± 8.52[c]
	IU01	211.76 ± 5.54[d]	527.23 ± 14.42[d]

ND* = represents not detected and DW* = represents dry weight. The values are expressed as the mean ± SD and different letters represent significant differences ($p < 0.05$).

Figure 1. Assessment of polyphenolic contents in plant exposed to Zn concentration (20 and 50 mg/kg). The means of the three values are presented as the mean ± SD and different letters on one treatment represent significant differences ($p < 0.05$).



used in the present study as an inoculum to enhance plant phytoremediation capability. The IU01 was grown in LB broth of for 72 h at 37°C and inoculum was used to plants treated with different concentrations 0, 20 and 50 mg/kg of soil. Whereas, 0 mg/kg of Zn was used as control to compare the treated and untreated results.

Estimation of growth attributes of ***B. napus:*** The experiment was harvested after 50 days and plant growth attributes of Zn treated and control plant was determined. The total length of the plants (Zn treated and control) was measured as root length and shoot lengths. Fresh biomass plant after harvesting was measured and then plants were dried at 100°C for 10 min and dried biomass was measure. The chlorophyll contents of the treated and control plants were also determined using chlorophyll meter (SPAD- 502, Minolta, Japan).

Zinc accumulation in ***B. napus:*** The oven-dried plant samples were crushed into powder using mortar and pestle and the 100 mg of powder were subjected to acid digestion. The solution, HNO3-$HClO_4$ was used to digest the sample powder. The concentration of Zn samples digested with acid was estimated through double plasma spectral analysis (ICP, PerkinElmer, USA).

Assessment of proline content: Proline contents of the plants under Zn stress and control were estimated through spectrophotometer at OD 765 nm. The plant was crushed in 0.5 mL methanol and the extract was added with 10% Folin-Ciocalteu reagent (2.5 mL). The mixture was dissolved in a solution of 7.5% Na_2CO_3 in 2 mL water. The blank was made as same without plant sample. The samples were incubated at 45°C for 45 min and absorbance was measured at 765 nm.

Statistics: The data were analyzed obtained from three independent experiments and analysis using Duncan's multiple range test.

RESULTS AND DISCUSSION

Estimation of growth attributes of ***B. napus:*** The plants grown under different Zn concentration i.e., 20 and 50 mg/kg and effects of Zn stress was determined on plant. The growth attributes such as chlorophyll contents, roots and shoot lengths, fresh biomass and dry biomass was determined as compared to 0 mg/kg of soil, used as control. The results revealed that Zn stress at 20 and 50 mg/kg significantly reduced ($p < 0.05$) the chlorophyll contents, roots length, and shoots length, dry biomass and fresh biomass as compared to control. However, inoculation of isolate IU01 improved the shoots length, roots length, chlorophyll contents, fresh and dry biomass as compared to plant exposed to different concentration of Zn as well as control (Table 1). The growth reduction compared to the control was evident at higher concentrations of Zn. The length of roots and shoots were significantly affected in a dose-dependent manner compared to the length of the control plants. Heavy metal resistant bacteria, e.g., Morganella sp., Providencia spp., and *Stenotrophomonas sp.*, were reported to have great bio-sorption and mobilization potential (Kartik et al., 2016). Previous studies have shown that bacterial endophytes were not only capable of detoxifying heavy metal toxicity but also promoting plant growth (Jabeen et al., 2009; Ullah et al., 2013; Fidalgo et al., 2011).

It has also been reported a Zn tolerant bacterial isolate, from different plants promoted plant growth and Zn tolerance in Brassica plants (He et al., 2013). Heavy metal resistant and plant growth promoting bacteria: *Azomonas sp.* RJ4, *Bacillus sp.* RJ16, Xanthomonas sp. RJ3, Bacillus sp. RJ31 and *Pseudomonas sp.* RJ10 have been reported to have effective in plant growth promotion and biomass enhancement in *B. napus* and *B. juncea* (Mata et al., 2002; Sheng et al., 2008; Ma et al., 2013; Wan et al., 2012; Liu et al., 2009; Kurzbaum et al., 2014).

Assessment of Zn accumulation in shoots and roots of the ***B. napus:*** The level of Zn accumulations in shoots and roots of the plants grown in 20 and 50 mg/kg of Zn were evaluated. The results revealed that the *B. napus* plant accumulated significantly higher concentration in roots then shoot. In addition, the inoculation of IU01 markedly improved the Zn accumulation the roots of *B. napus* (Table 2). The accumulation of heavy metals in parts of plants i.e., shoot and roots has presumably attributed to the extensive

root of the plants. Biomass increase through endophytic bacteria confined to the roots of the plants (Sheng et al., 2008). Same results of Zn accumulation were also reported by Wei et al. (2013), their report showed that an increase in Zn in the soil was associated with higher amounts of Zn accumulating in roots of the *B. napus*. The studies conducted by Malandrino et al., (2006), Wan et al., (2012) showed that Zn accumulation was higher in the roots of plant as compared to shoots. Similarly Wan et al. (2012) reported that the level Zn accumulated in the roots was many-fold higher as compared to the shoots of the *B. napus*.

Effects Phenolic contents on plant under Zn stress: The results revealed that high concentration of Zn markedly increase the phenolic content of plants treated with 20 and 50 mg/Kg of Zn as compared to control. The IU01 inoculation significantly ($p < 0.05$) decrease the total phenolics in plants stressed with Zn various concentration such as 20 and 50 mg/kg as compared to uninoculated plant (Fig. 1). Zinc stress in plants was neutralized by phenolic contents produced by plants under the plant defense system (Ahmad et al., 2014; Hossain et al., 2012). Application of strain IU01 on *B. napus* plant treated with different Zn concentration, mediated the antioxidant activities significantly ($p < 0.05$) as compared to uninoculated plants. Previously, it has been reported that phenolic contents in plants were increased when exposed to high level of heavy metal; however, application of IU01 significantly reduced the phenolic contents (Wang et al., 2008). Moreover, the reduction antioxidant activities was presumed to be because of increased biomass in bacterial inoculated plants (Ullah et al., 2019).

CONCLUSION

Zinc is a toxic heavy metal to plants and animals including human being even at low concentration. The Zn contamination has been a main problem in recent decades. Phytoremediation mediated ny bacteria is among the most important strategy through which the heavy metals are eliminate from the soil. In the present study, B. napus inoculated with IU01 was used in Zn contaminated soil. The IU01 was assessed to enhance the Zn accumulation predominantly in roots.

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Low-Cost Gravitational Force Based Colorimetric Microfluidic Device for Assaying Blood Glucose

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ABSTRACT

In this research was developed low-cost gravitational force based colorimetric microfluidic device for diagnostic purpose. Microfluidic system was developed by using discarded pen refills. The refill was filled with three layers of polymer. Bottom of the refill wasfilled with the polymer polyvinyl chloride (PVC). Second layer from the bottom i.e the layer above PVC layer wasfilled with silica gel immobilized with horse radish peroxidase (HRP), glucose oxidase (GOD) and o- dianisidine (dye). Whereas, third and the top most layer wasfilled with untreated silica gel. One drop of blood waspoured at the inlet of microfluidic devices. Without applying any external power, blood moved through the silica packed region by the gravitational pull and capillary action of silica gel. Serum separation started within 30 seconds and subsequently within 2 min., serum successfully separated from the blood by pure silica gel. The separated serum then came in contact with the silica gel immobilized with enzymes and dye. The colour of the silica beads immobilized with enzymes and dye changed from white to orange when came in contact with glucose in serum. Determination of the glucose in the blood was carried out on a desktop scanner. The developed microfluidic device does not require (i) pump or device to propel the fluid (ii) any type of special mesh or sieve to separate the serum from the blood. Microfluidic device developed is cheap and suitable for low cost setting areas.

KEY WORDS: GRAVITATIONAL FORCE-BASED MICROFLUIDIC DEVICE, POINT-of-CARE DIAGNOSTICS, BLOOD GLUCOSE ASSAY, SILICA GEL, LOW COST.

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INTRODUCTION

The field of microfluidics is characterized by the study and manipulation of fluids at the submillimetre length scale. Most of the microfluidic devices often require one or more pumps to propel fluid through microchannels or additional power supply as driving force (Araci and Quake, 2012) which makes the system quite expensive and complicated. Gravitational pulls based microfluidic devices are cheap and simple to operate (Huh et al., 2007). However, these devices are hard to develop since they are based on only gravitational force. Rhee et. al. have described the hydrodynamic and gravitational force-based positioning of cells within microfluidic devices that can be implemented without special equipment or fabrication steps (Rheem et al., 2007). Yao et al. (2004) have developed a gravity and electric force driven microfluidic device for cell sorting (Yao et al., 2004).

Most of the work related to gravitational pull based microfluidic device reported till now are mainly related to the area of cell sorting and particle separation. There are hardly any reports on the gravitational pull based microfluidic devices, which are used for detecting glucose in the blood, (Sackmann et al., 2014).

Figure 1. Schematic illustration of packed silica beads and blood/ serum separationfor Proposed Method

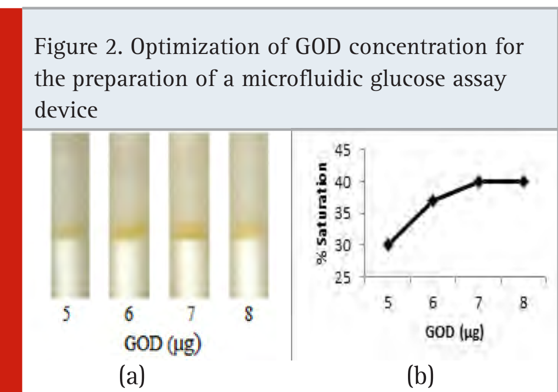
Diabetes is one of the principal causes of death and disability in the developing world and is highly responsible for heart disease, kidney failure, and blindness. It is a major cause of mortality in the age group of 20–79 years. Based on its rapidly increasing incidence, diabetes has been declared a global epidemic by the World Health Organization (WHO) (Derek et al., 2006). Frequent testing of physiological blood glucose levels to avoid diabetic emergencies is crucial for the confirmation of effective treatment. There have been continuously increasing research efforts in the field of glucose monitoring during the last few decades. The frequent monitoring of blood glucose is critical for diabetic management, as the maintenance of physiological glucose level, i.e., 4–8 mM (72–144 mg/dL), is the only way that a diabetic can lead a healthy lifestyle by avoiding life-threatening diabetic complications, such as diabetic retinopathy, kidney damage, heart diseases, stroke, neuropathy and death.

The most widely used glucose monitoring devices are blood glucose meters which are based on minimally-invasive fingerstick tests that have a substantial market. Several continuous glucose monitoring systems (CGMS), which are presently more than five years old, can also detect glucose at low levels (Bode et al., 2008). Most of the CGMS employ minimally-invasive approaches that involve the use of subcutaneous sensors to determine the glucose concentration in interstitial fluid. Therefore, they cause discomfort to patients, require more frequent calibration by fingerstick tests and cannot be used for more than a few days, as the sensor is prone to biofouling. However, the main limitation is their extremely high cost, which is beyond the reach of most diabetics. Besides this, it also requires calibration with blood glucose testing and change of the sensor after a few days. This strictly limits their use to only selected clinical scenarios, where they are critically required. Glucose oxidase (GOD)-based glucose biosensors have prevalently held the glucose sensor research and development over the last four decades and the market place as

well. This is due to the good stability of GOD that makes the glucose/glucose oxidase system a very convenient model for glucose detection. There is a high demand for sensitive and reliable blood glucose monitoring in biological and clinical aspects. However, the high cost of glucose sensors available in the market is one of the main disadvantages of enzyme-based glucose determination. Therefore, the development of a low-cost detection system that can reduce the operational cost, calibration and warm-up period has been the subject of concern. This paper reports a silica gel based on a low-cost glucose detection system in discarded pen refills. Silica serves as a well-established dispersing material for proteins because it is non-toxic, chemically and biologically inert, subject to negligible hydration swelling, hydrophilic, and inexpensive to synthesize (Nassif et al., 2003; Chiriac et al., 2010).In the present work, a low cost colorimetric microfluidic device for detection of glucose was developed. Capillary force has driven microfluidic system was developed by using discarded pen refills of 2.66 mm width and 4 cm height.

Pen refills are generally made up polypropylene polymer. Polypropylene is inert and is insoluble in most of the organic solvent, hence is suitable for the present work. Refill was packed with three layers of polymer. Bottom of the refill was filled with polyvinyl chloride (PVC) 20 mg to prevent the leakage of fluid from the refill. The strong and linear nature of the polymer prevents the dispersion of the liquid from the loaded refill. Second layer i.e the layer above PVC layer was filled with 5mgof treated silica gel i.e silica gel immobilized with Horseradish peroxidase (HRP), Glucose Oxidase (GOD) and o- dianisidine (dye), henceforth named as treated silica. Whereas, third and the topmost layer was filled with pure silica gel. In the present study, pure silica gel was used to separate the serum from the blood within a short period of time. Whereas treated silica was used for detecting glucose in the blood. Glucose oxidase oxidizes glucose into D- glucono -1,5 lactone with the formation of hydrogen peroxide. Horseradish peroxidase enzyme further breaks hydrogen peroxide to water and oxygen. Oxygen then reacted with an oxygen acceptor dye (OPD) which itself converted to a coloured compound, the amount of which can be measured colorimetrically. To check the workability of the device, glucose was detected in the blood samples. Four microliters of freshly drawn human blood was carefully dropped at the inlet of the microfluidic device. Without applying any external power, blood moves through the silica packed region by capillary force. The movement of blood cells was impeded by small pores between the packed-beads. The hydrophilic surface of the packed beads induced the capillary flow of serum. After dropping 4µl of whole human blood, the serum separation started within 30 seconds and serum was successfully separated within 2 minutes by the silica gel.

Figure 2. Optimization of GOD concentration for the preparation of a microfluidic glucose assay device



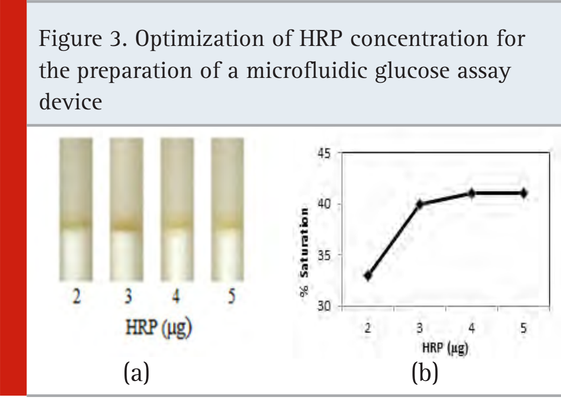
The separated serum then came in contact with the treated silica beads and the colour of the treated silica beads changed from white to orange when in contact with glucose in serum (Fig. 1). Determination of the glucose in the blood was carried out on a desktop scanner (HP photo smart C6388) by placing the tube in the scanner to get the image. From Adobe Photoshop the mean value of each R, G and B of the scanned image was obtained which was then converted to HSB (Hue, Saturation, and Brightness) using freely available "Macbeth colour calculator" software. The image was then quantified as the saturation percentage. We have termed "saturation" as "colour-saturation" to make it clear as well as to differentiate it from a conventional chemical understanding of saturation (Parween and Nahar, 2013).

MATERIALS AND METHODS

Horse radish peroxidase (HRP) was purchased from Sigma (USA). Glucose Oxidase (GOD) was purchased from Boehringer Mannheim GmbH, Silica was purchased from SiscoResearch Laboratories Pvt. Ltd., D- Glucose, o-dianisidine (dye) and polyvinyl chloride (PVC) were of analytical grade and purchased either from SRL, Glaxo or Merck (India). All buffer solutions were freshly prepared in triple distilled water before use. Phosphate Buffered Saline (PBS) was prepared by mixing 0.85 % NaCl to 0.01 M phosphate buffer (pH 7.2). A 1.5mg/ ml stock solution of enzyme HRP and GOD was prepared in PBS and stored in the refrigerator for further use. Citrate-buffer (pH 5) was prepared by mixing 0.025 M citric acid and 0.05 M di-sodium-hydrogen phosphate in 100 ml distilled water. o-dianisidine (dye) was prepared in Citrate buffer and Glucose solution was prepared in distilled water.Human blood was obtained from a healthy volunteer as well as from diabetic one and Blood sugar was checked with a Blood Glucose meter (ACCU-CHEK).

Microfluidic device fabrication: The microfluidic device was made from discarded pen refill. Gravity-driven microfluidic system was developed by using discarded pen refills having 2.66 mm width and 4 cm height. Refill was washed with acetone to remove the ink remaining in the refill and dried. Bottom of the refill was sealed with parafilm at the end to prevent the leakage of the liquid. A 20 mg of PVC was filled at the bottom of the refill to prevent the diffusion of colour. Above the PVC layer,10 mg dried treated silica was loaded and subsequently, 40 mg of pure silica was loaded onto the treated silica. Refills were then ready for glucose estimation. Optimization of the amount of Glucose Oxidase (GOD) for immobilization onto the pure silica gel to get treated silica gel: Petri plate containing 50 mg pure silica gel was taken and mixed with HRP (20 µg/ 50 µl PBS)and dye (400 µg/50 µl buffer). This mixture was divided into 4 parts each having 10 mg of the mixture. Four parts of the prepared mixture were taken and varying amounts of GOD corresponding to 5, 6, 7 and 8 µg/10 µl PBS was added respectively to get treated silica. Treated silica gels were then kept for drying in the dark. Four clean refills were taken and one end of each refill was closed with parafilm. PVC (20 mg/ refill) was then filled in the refill as a bottom layer of the device. Then, 10 mg of treated silica containing varying amounts of GOD was loaded into the refill separately. Subsequently, 40 mg of pure silica was loaded onto the treated silica to get four separate refills containing varying amount of HRP loaded refill. To get the optimum amount of GOD, 4 µl of glucose solution (200 mg/dl) was poured into each refill and subsequently PBS (76 µl) was poured into each refill (Fig. 2a). After 8 minutes, the refills were scanned on a desktop scanner (HP photo smart C6388) to get an image. From Adobe Photoshop, the mean value of R, G and B of the scanned image was obtained which was then converted to HSB (Hue, Saturation, and Brightness) using freely available “Macbeth colour calculator” software. The image was then quantified as the saturation percentage.

Figure 3. Optimization of HRP concentration for the preparation of a microfluidic glucose assay device

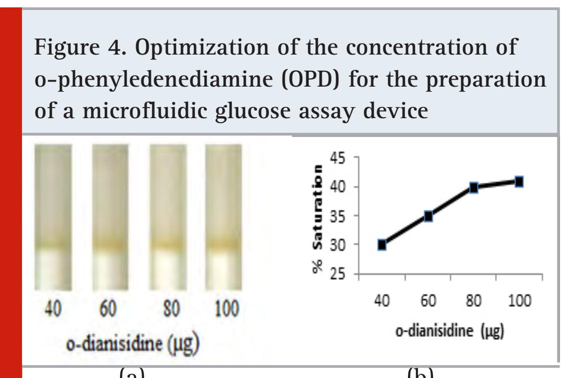


Optimization of the amount of HRP for immobilization onto treated silica gel from pure silica gel: Petri plate containing 50 mg pure silica gel wastaken and mixed with GOD (35 µg/50 µl) and dye (400 µg/50 µl). This mixture was divided into 4 parts each having 10 mg of the prepared mixture. Four parts were taken and varying amounts of HRP corresponding to 2, 3, 4 and 5 µg/ 10 µl PBS buffer was added separately and respectively. These mixtures were then kept for drying in dark.Four clean refills were taken and one end of each refill was closed with parafilm. PVC (20 mg/refill) was then filled in the bottom layer of the refill. 10 mg of treated silica with varying amount of HRP was loaded into the refill separately. Subsequently, 40 mg of

pure silica was loaded onto the silica mixture to get four separate fully loaded refill of varying HRP concentration. To get the optimum amount of HRP, 4 µl of glucose solution (200 mg/dl) was poured to each refill and subsequently PBS (76 µl) was poured into each refill (Fig. 3a). Fig 3: Optimization of HRP concentration for the preparation of a microfluidic glucose assay device. After 8 minutes, the refills were scanned on a desktop scanner (HP photo smart C6388) to get the image and saturation percentage was calculated as described above.

Optimization of the amount of o-phenyledenediamine (dye) for immobilization onto silica gel: Petri plate containing 50 mg pure silica gel was taken and mixed with GOD (35 µg/50µl) and HRP (20 µg/50 µl). This mixture was divided into 4 parts each having 10 mg of the prepared mixture. Four parts were taken and varying amounts of dye corresponding to 40, 60, 80 and 100 µg/10 µl citrate buffer was added. Four Petri plates containing mixtures were then kept for drying in dark.Four clean refills were taken and one end of each refill was closed with parafilm. PVC (20 mg/refill) was then filled in the bottom layer of the refill (device). 10 mg of dried treated silica with varying amount of dye was loaded into each refill respectively. Subsequently, 40 mg of pure silica was loaded onto the silica mixture to get a fully loaded refill. For detection, 4 µl of glucose solution (200 mg/dl) was poured into each refill and subsequently PBS (76 µl) was poured into each refill (Fig. 4a). After 8 minutes, the refills were scanned on a desktop scanner (HP photo smart C6388) to get the image and saturation percentage was calculated as described above.

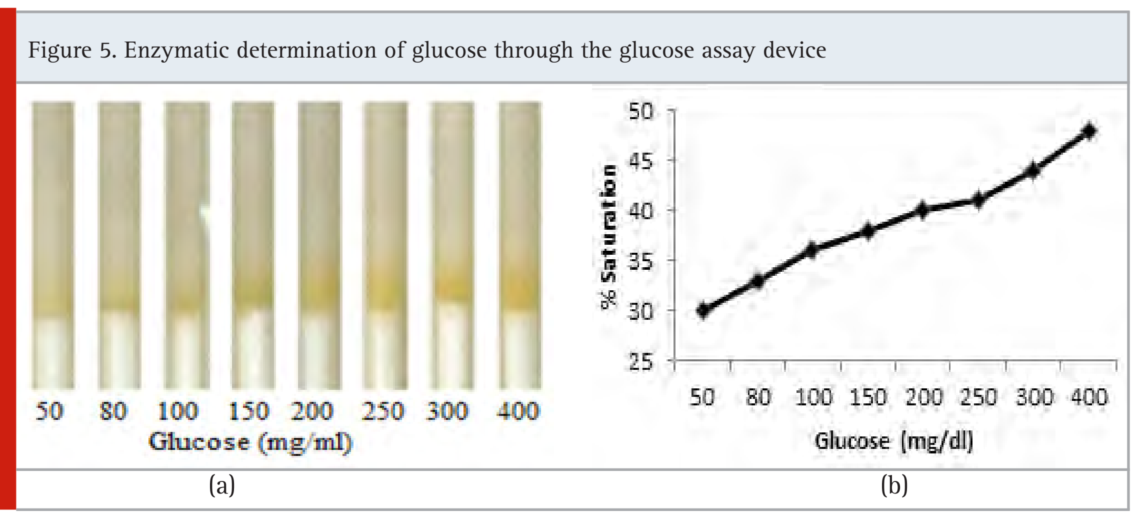
Figure 4. Optimization of the concentration of o-phenyledenediamine (OPD) for the preparation of a microfluidic glucose assay device



Determination of glucose in a synthetic sample: Treated silica was prepared by adding HRP (40 µg/100 µl), GOD (70 µg/100 µl) and dye (800 µg/100 µl) with 100 mg of pure silica gel. Treated silica was filled into the different tubes as described in earlier sections.Four µl of varying concentration of D-glucose (50, 80, 100,150, 200, 250, 300 and 400 mg/dl) wasadded to each refill and subsequently, PBS (76 µl) was poured into each refill (Fig. 5a). After 8 minutes, the refills were scanned on a desktop scanner (HP photo smart C6388) to get the image and saturation percentage was calculated as described above.

Determination of Glucose in blood samples with added synthetic glucose: Treated silica was

Figure 5. Enzymatic determination of glucose through the glucose assay device



prepared by adding HRP (40 µg/100 µl), GOD (70 µg/100 µl) and dye (800 µg/100 µl) with 100 mg of pure silica gel. Treated silica was filled into different tubes as described in earlier sections. Fresh blood sample from a fasting individual was taken and glucose level was measured in a blood sample with a digital glucose meter which was found to be 80 mg/dl. Varying concentrations of synthetic glucose (0, 20, 70, 120, 170, 220, and 320 mg/dl) was added in 4 µl of blood sample to get seven samples of varying concentration containing 80, 100, 150, 200, 250, 300, 400 mg/dl glucose respectively. For detection, to each refill, 4 µl of prepared samples were poured and subsequently, PBS (76 µl) was poured into each refill (Fig. 6a). After 8 minutes, the refills were scanned on a desktop scanner (HP photo smart C6388) to get the image and saturation percentage was calculated as described above.

Determination of Glucose in blood samples without adding synthetic glucose: Treated silica was prepared by adding HRP (40 µg/100 µl), GOD (70 µg/100 µl) and dye (800 µg/100 µl) with 100 mg of pure silica gel. Treated silica was filled into different tubes as described in earlier sections. Four µl of a blood sample from three individuals, (i) from fasting individual (ii) from non- fasting individual (iii) from a diabetic individual, was poured into each refill respectively for detection and PBS (76 µl) was subsequently poured into the refills (Fig. 7a). After 8 minutes, the refills were scanned on a desktop scanner (HP photo smart C6388) to get the image and saturation percentage was calculated as described above.

Determination of R.G.B. values: The colour formed after the reaction was scanned with a desktop scanner. The scanned image was opened in Adobe Photoshop Version 7.0. The area of colour was selected using a rectangular marquee. After the region was selected, the median of R.G.B. values for each refill was noted.

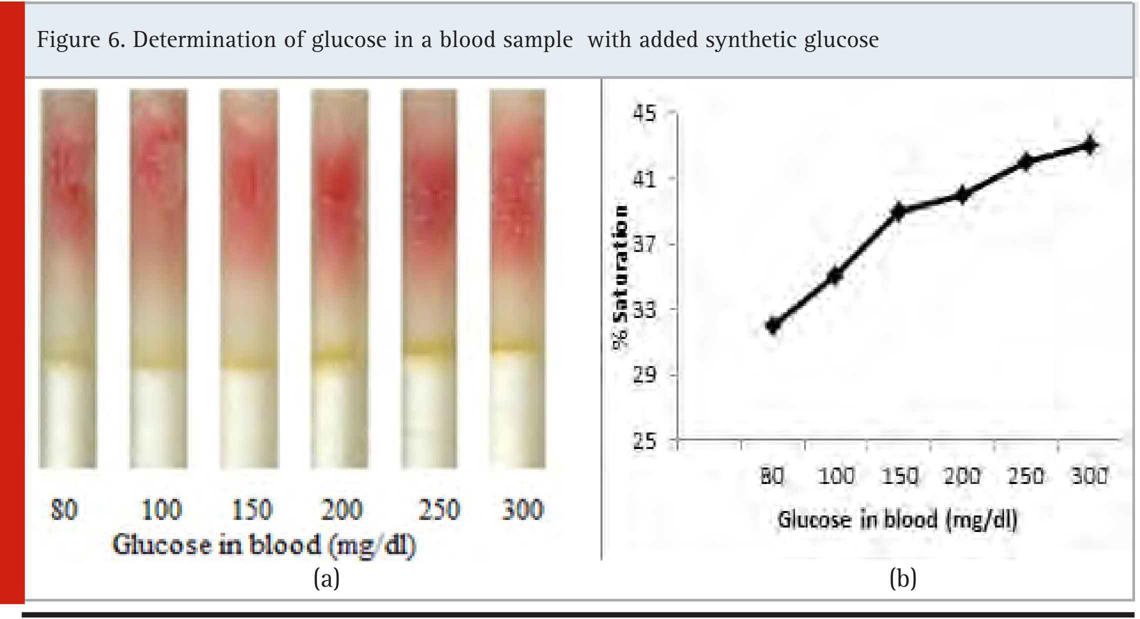
Determination of Saturation: Saturation of each reading was calculated by Macbeth calculator. This is used for calculating hue, saturation and brightness. Saturation was calculated by providing R.G.B. values calculated from Adobe Photoshop. Saturation was noted down for each refill.

Preparation of graphs based on concentration and saturation values: Graphs were prepared by plotting saturation percentage depending on the colour obtained against their concentrations as shown in Figs. 2b, 3b, 4b, 5b, 6b and 7b.

RESULTS AND DISCUSSION

The most common way to check glucose levels involves pricking a fingertip with an automatic

Figure 6. Determination of glucose in a blood sample with added synthetic glucose



lancing device to obtain a blood sample and then using glucose strips of glucose meter to measure the blood sample's glucose level. Glucose test strips of glucose meter are expensive due to expensive enzymes, precious metals, chemicals, and other materials that makeup test strips.We proposed gravitational force driven and capillary action based low-cost microfluidic device for assaying blood glucose. The proposed microfluidic device wasmade out of discarded pen refills of 2.66 mm width and 4 cm height. Pen refills are generally made up polypropylene polymer which is inert in nature and is insoluble in most of the organic solvent. Pen refills filled with the polymer viz. silica, was utilized as a multipurpose polymer i.e. it wasused for separating sera from blood as well as used for detecting glucose when GOD was immobilized onto it.

Silica serves as a well-established dispersing material for proteins because it is non-toxic, chemically and biologically inert, subject to negligible hydration swelling, hydrophilic, and inexpensive. In the present study, pure silica gel was used to separate the serum from the blood within a short period of time [14]. Whereas treated silica i.e silica gel immobilized with GOD, HRP and dye was used for detecting glucose in the blood. Glucose oxidase oxidized glucose into D- glucono -1,5 lactone with the formation of hydrogen peroxide. Horseradish peroxidase enzyme further broke hydrogen peroxide to water and oxygen. Oxygen then reacted with an oxygen acceptor dye (OPD) which itself was converted to a coloured compound, the amount of which can be measured colorimetrically. Figure 1 shows the Schematic illustrations of packed silica beads and blood/serum separation. Without applying any external power, blood moved through the silica packed region by capillary force and serum separation started within 30 seconds and successful serum separation took place within 2 min of loading. Enzymatic determination of Glucose onto the treated silica gel was carried out and was noted that the treated silica gel (silica immobilized with GOD, HRP and dye) could detect the glucose concentration successfully. In order to obtain an optimum amount of HRP, GOD and dye and to get best treated silica gel, three separate experiments were carried out. As shown in Figure 2, an optimum amount of GOD required to prepare treated silica gel was0.7 µg per 50 mg silica gel, whereas the optimum amount of HRP required iswas0.4 µg per 50 mg silica (Figure 3). O-dianisidine (dye) required to act appropriately with the optimum amount of HRP and GOD was found to be 80 µg per 50 mg silica (Figure 4).

Figure 5 shows the enzymatic determination of glucose onto the treated silica gel (silica immobilized with GOD, HRP and dye). Standard plot was made using varying glucose concentration. It is noted from Figure 5 that thepercentage (%) saturation increases with an increase in glucose concentration.Determination of Glucose in the blood sample with added synthetic D-glucose was further carried out. In this experiment, blood sample was taken from fasting individual and glucose level waschecked with a digital glucose meter.

The blood glucose level in the fasting individual was found to be 55 mg/dl. Four microliter of blood sample was taken from the same fasting individual (with glucose level 55 mg/dl) in seven different eppendorf and varying glucose concentration of 25, 45, 95, 145, 195, 245 and 345 mg/dl was added in the blood sample to get seven sample of varying concentration of 80, 100, 150, 200, 250, 300, 400 mg/dl respectively. These prepared samples were then poured in the loaded pen refills and % saturation was determined (Fig. 6). On comparing the % saturation of Figure 5 (wherein the % saturation of only synthetic glucose is demonstrated and standard glucose

Figure 7. Determination of glucose in the blood sample without adding synthetic glucose

91 126 185
Glucose in blood (mg/dl)
(a)

Saturation %
Glucose in blood (mg/dl)
(b)

plot is made) with that of Figure 6, it is very clear that the amount of sugar can be calculated using Figure 4, since the results are comparable. Further, we also detected the blood glucose level in three individual, where we did not add any synthetic sugar in human blood (Fig. 7). In this case we took a blood sample from (i) fasting individual (ii) non- fasting individual (iii) the diabetic individual. It is very clear from Figure 7 that the detection of the sugar can be carried out with the help of a glucose standard plot (Figure 5), since the results are comparable.

CONCLUSION

A simple, rapid and economical microfluidic device for assaying blood glucose level was developed. The developed glucose detection system is far simpler and cheaper than the systems/machine available in the market. With minor modifications in the treated silica region, the device can be used in other areas of diagnostics also.

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In vitro Phytochemical Profiling of Anti Diabetic and Anti Inflammatory Activities of Premna rajendranii – An Endemic Species

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ABSTRACT
The present study aims to find out the presence of phytochemicals and evaluate the anti diabetic and anti-inflammatory activities of *Premna rajendranii*.Anti diabetic assay was evaluated using α- amylase inhibitory assay by DNSA method, anti-inflammatory activity was tested using the method of Mizushima et al with simple modifications and the methods of Peach and Tracey (1956), Gibbs (1974), Harborne (1984), Trease and Evans (1985), Edeoga et al.,(2005), Khandewal (2008), Kokate et al.,(2001), Sofowara (2009) and Tiwari et al.,(2011) were used to identify the nature of phytochemical constituents present in *Premna rajendranii*.Anti diabetic analysis of methanolic leaf extract of *Premna rajendranii* showed optimal activity when compared to standard drug. Maximum inhibition showed at 100µg (79.3%) and minimum inhibition showed at 25 µg (44.82%). Anti-inflammatory activity of methanolic leaf extract of P.rajendranii has shown high inhibition than standard at 50µl i.e. 33.88±0.01. Phytochemical screening shows the presence of Alkaloids, Carbohydrates, Flavonoids, Steroids, Glycosides, Phenols, Proteins, Tannins, Saponins, and Terpenoids in the leaf of *Premna rajendranii*.Anti diabetic and anti-inflammatory analysis showed the presence of bioactive compounds and the medicinal values of *Premna rajendranii*.

KEY WORDS: *PREMNA RAJENDRANII, ANTI DIABETIC, ANTI-INFLAMMATORY, PHYTOCHEMICAL ANALYSIS.*

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INTRODUCTION

Plants have an important role in the day to day life of mankind. Each and every plant contains phytocompounds either with medicinal properties or poisonous properties. Scientists and researchers are more focused on medicinal plants for the benefit of mankind. According to them each of the medicinal plant is a source of drug candidate. From ancient times India is considered a rich repository of medicinal plants and Indian traditional systems of medicine such as Ayurveda, Siddha and folk medicine and well known. Genus *Premna* has an indispensable value in Indian health care practice. Species of *Premna* are known for the preparation of famous Ayurvedic formulation Dashamoolaarishtam (Joshi et al., 2017). *Premna's* medicinal properties have been used in Indian traditional system of medicine especially for diarrhoea, stomach and hepatic disorders. The various biological activities including antioxidant, antibacterial, anti-inflammatory, cytotoxic and heapatoprotective have been displayed both at extract and pure compound level (Rekha, 2015). During the taxonomic revision of Indian Verbenaceae, Rajendran and Daniel (2002) recognized 31 species and 6 varieties of *Premna*. Recently, PrabhuKumar et al.

(2013) reported the discovery of a new species *Premna rajendranii* from Western Ghats (Chinnar of Kerala and Madukkarai of Tamilnadu). Apart from this, a research team comprising Robi, Augustin, Sasidharan and Udayan (2013) rediscovered an endemic and rare species *Premna paucinervis* (C. B. Clarke) Gamble from the Vagamon hills along South Western Ghats of Kerala after a lapse of 140 years of its original type collection by R.H. Beddome (1872) from the Anamalayas in the Western Ghats (Tamilnadu) (Bose, 2014). Premna included earlier in the family Verbenaceae, was recently transferred to the Lamiaceae family based on molecular data (A.P.G. IV. 2009; Francis et al., 2019).

MATERIALS AND METHODS

Collection and authentication: The plant material for our investigation was collected from the scrub jungles of the Madukkarai Hills, Coimbatore District in Tamilnadu, and authenticated by Dr. S. John Britto S.J, at the Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Autonomous), Tiruchirappalli. The voucher Specimen (RHT 68887) was deposited in the Rapinat Herbarium.

Extraction: The leaves were shade dried and powdered using mechanical grinder. The powder sample was stored in an air tight container and the portion of the powder was taken in test tube and solvent (Methanol) was added to it such that plant powder soaked in it and shaken well. The solution then filtered with the help of muslin cloth and filtered extract was taken and used for antidiabetic and anti-inflammatory analysis.

Preliminary Phytochemical Analysis: Test's for Alkaloids: Mayer's Test: To a few ml of filtrate, one or two drops of Mayer's reagent were added by the side of the test tube. A white creamy precipitate indicated the test as positive. Wagner's Test: To a few ml of filtrate, few drops of Wagner's reagent were added by the side of the test tube. A reddish brown precipitate confirmed the test as positive. Hager's Test: To a few ml of filtrate 1 or 2 ml of Hager's reagent (Saturated aqueous Solution of picric acid) was added. A prominent yellow precipitate indicated the test as positive. (Kokate et. al., 2001).

Test for flavonoids:Pews Test: To 2-3ml extract, was added zinc powder in a test tube, followed by drop wise addition of conc. HCL. Formation of purple red or cherry colour indicated the presence of flavonoids (Peach et.al., 1956). Lead acetate test: 1ml extract was treated with 1ml 10% lead acetate (Pb(OAc)4) solution. Formation of yellow colour precipitate indicated the presence of flavonoids. Alkaline reagent test: To 2ml test solution, sodium hydroxide solution was added to give a yellow or red colour (Khandewal et.al. 2008). Conc.H_2SO_4 test: 5ml of dilute ammonia solution was added to the extract followed by conc.H_2SO_4. Yellow colour indicated the presence of flavonoids. Tests for Phenolic Compounds and Tannins:Ferric Chloride Test: The extract (50 mg) was dissolved in 5 ml of distilled water. To this few drops of neutral 5% Ferric Chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

Potassium dichromate test: To the extract add 5% potassium dichromate solution. Positive result was confirmed by a formation of brown precipitate (for phenol).

Lead Acetate Test: The extract (50 mg) was dissolved in distilled water and to this 3 ml of 10% Lead Acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds (Treare et.al., 1985). Braymer's Test: To 2 ml extract, added 2 ml H2O and followed with 2-3 drops of FeCl3 (5%). Green precipitate proved presence of tannins. Tests for Saponins: Foam Test: To 1ml of extract, add 2ml of distilled water and shaken vigorously and allowed to stand for 10 min. There is the development of foam on the surface of the mixture. Then shake for 10 minutes, it indicates the presence of saponins (Khandewal et.al.,2008). $NaHCO_3$ Test: To extract a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 min. A honey comb like froth was formed and it showed the presence of saponins. Tests for Glycosides: Keller Kiliani Test (Test for cardiac glycoside): To 2 ml extract, was added 1 ml glacial acetic acid, one drop 5% $FeCl_3$ and 1 ml conc. H_2SO_4 . A brown ring of the interface indicated the presence of cardiac glycosides (Kokate et.al., 2001; Khandewal et.al., 2008). Glycoside Test: To small amount of extract, was added 1 ml water and shake well. Then aqueous solution of NaOH was added. Yellow color appeared that indicated the presence of glycosides (Treare et.al., 1985).

Molisch's Test: To 1ml of extract, 2drops of Molisch's reagent was added in a test tube and 2ml of con. H_2SO_4 was added carefully keeping the test tube slightly curved. Formation of violet ring at the junction indicated the presence of glycosides (Khandewal et.al., 2008).

Tests for Carbohydrates: Molish's Test: To 2 ml of filtrate two drops of alcoholic solution of α- napthol was added, the mixture was shaken

Table: 1.1 Preliminary phytochemical analyses in Premna rajendranii (leaf)

	Test	Chloroform	Acetone	Ethanol	Methanol	Aqueous
Alkaloids	Wager's	+	+	++	+++	++
	Hager's	_	+	++	+++	++
	Mayer's	_	+	++	+++	+
Flavonoids	Pew's	+	+	+	++	_
	Lead Acetate	+	+++	+++	+++	+++
	NaOH	_	+++	+++	+++	+++
	Con.H_2SO_4	+	+++	+++	+++	++
Phenol & tannin	$FeCl_3$	+	+	+	++	+
	$K2Cr_2O_7$	+	+	+	+	+
	Lead Acetate	_	_	+	++	+
	Braymers	+	_	+	+	+
Saponins	Foam	_	_	_	_	_
	$NaHCO_3$	_	_	_	_	_
Glycosides	Keller kiiani	_	+++	+++	+++	+++
	Glycosides	_	+++	+++	+++	+++
	Molish	_	++	+++	+++	++
Carbohydrates	Molish	_	+	++	++	++
	Benedicts	_	_	+	+	_
Terpenoids	Salkowskis	_	+	+	++	+
Quinones	Quinones	_	_	_	++	+++
Sterols	Salkowskis	_	_	_	_	_
	Keller kiiani	_	+	+	++	++
Protein	Biuret	_	+	+	+	+

well and 1 ml of con. H2SO4 was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates. Benedict's test: To 0.5 ml of filtrate, 1 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 mins. A characteristic coloured precipitate indicated the presence of sugar. Test for Terpenoids:Salkowski's Test: 2 ml of chloroform and 1 ml of conc. H2SO4 was added to 1 ml of extract and observed for reddish brown color that indicated the presence of terpenoids. Tests for quinones: 1ml of extract was treated with alcoholic potassium hydroxide solution. Quinines give coloration ranging from red to blue. Test for sterols: Salkowski's Test: To 2 ml of extract, was added 2 ml chloroform and 2 ml conc. H2SO4 from the side of the test tube. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence indicated the presence of sterols (Khandewal et.al., 2008).

Table 2. Antidiabetic Activity of Methanolic leaf extract of *Premna rajendranii*

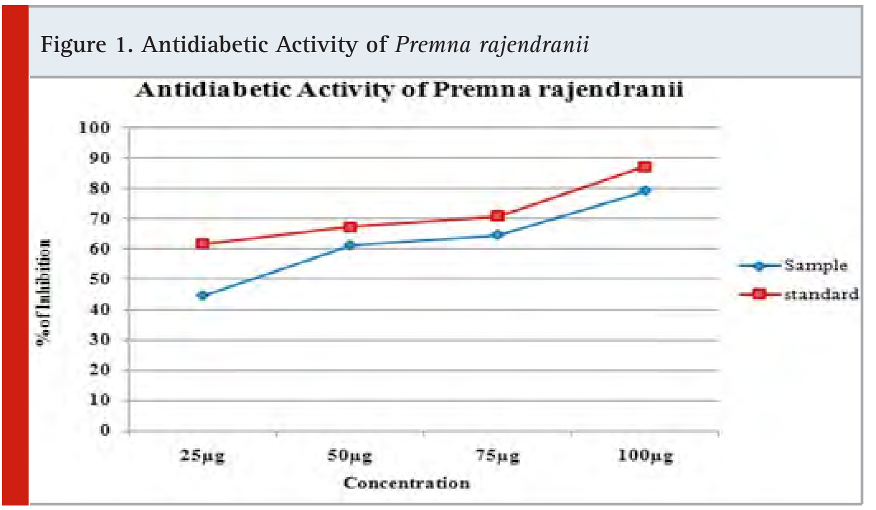
SL.NO:	Concentration in (µg)	% of inhibition	
		Sample	Standard
1	25	44.82±0.5	61.7±0.01
2	50	61.37±0.6	67.3±0.01
3	75	64.82±0.4	70.9±0.01
4	100	79.3±0.7	87.4±0.21
IC_{50} value	288.8	246.7	

Tests for Proteins and Amino Acids:Biuret Test: An aliquot of 2 ml of filtrate was heated with 1 drop of 2 % H_2SO_4solution. To this 1 ml of ethanol (95%) was added, followed by excess of KOH Pellets. Pink colour in the ethanolic layers indicated the presence of proteins. Conc. H_2SO_4Test: 2 ml extract was treated with few drops of conc. H_2SO_4. Formation of white precipitate indicated the presence of proteins.

Xantho proteins Test: 2 ml extract was treated with few drops of conc. HNO_3 and NH_3 solution. Formation of reddish orange precipitate indicated the presence of xantho proteins.

Anti-Diabetic Assay (A-Amylase Inhibitory Assay) By Dnsa Method: α-amylase was dissolved in phosphate buffer saline (PBS, 0.02 mol/L, pH6.8) at a concentration of 0.1 mg/ml. Various concentrations of sample solutions (0.25 ml) were mixed with α-amylase solution (0.25 mL) and incubated at 37 °C for 5 min. Then the reaction was initiated by adding 0.5 mL 1.0% (w/v) starch substrate solution to the incubation medium. After incubation at 37 °C for 3 min, the reaction was stopped by adding 0.5 ml DNS reagent (1% Dinitrosalicylic acid, 0.05% Na2SO3 and 1% NaOH solution) to the reaction mixture and boiling at 100 °C for 5 min. After cooling to room temperature, the absorbance (Abs) at 540 nm was recorded by a spectrophotometer. The inhibition percentage was calculated by the following equation:

Figure 1. Antidiabetic Activity of *Premna rajendranii*



Percentage Inhibition = [(Abs1 – Abs2)/Abs1] × 100

Where, Abs1= control and Abs2= sample.

Anti-Inflammatory Activity: Method of Mizushima et al was followed with simple modifications. The reaction mixture (0.5 ml) consisted of 0.45 ml bovine serum albumin (3% aqueous solution) and varying concentration of compound (25, 50, 100,200µg/ml of final volume), pH was adjusted to 6.3 using small amount of 1N hydrochloric acid. The samples were incubated at 37°C for 20 min and then heated at 80°C for 2min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The absorbance was measured using spectrophotometer at 660nm.The percentage inhibition of protein denaturation was calculated as follows:

Percentage of inhibition = [(Abs Control – Abs Sample) / Abs control)] x100

RESULTS AND DISCUSSION

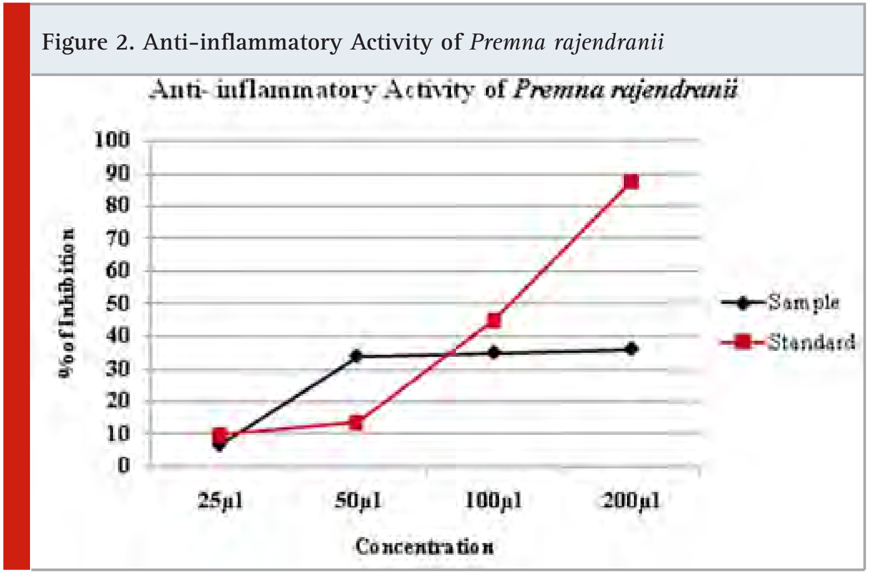
The result of phytochemical profiling of leaves of *P.rajendranii* indicates the presence of Alkaloids, Flavonoids, Phenols, Tannins, Glycosides, Carbohydrates, Terpenoids, Quinones, Sterols and Proteins. High concentrations of bioactive compounds were found in methanolic, ethanolic, aqueous, acetone extracts while very low concentrations in chloroform extract. Alkaloids, Flavonoids and Glycosides were mainly seen in most of the samples except chloroform extraction (Table 1).

Diabetes mellitus is one of the major metabolic disorders (Keerthana et al., 2013), and is characterized by high blood glucose levels. According to World Health Organization, 180 million people are currently suffering from DM and the rate is rapidly growing. There are many effective medicines for DM but with serious side effects. Recently researchers are in the experiments to find effective medicine for DM without any side effect. More than 1200 plants have a potential antidiabetic capacity (Modak et. al., 2007; Bailes, 2002; Chitturi and George, 2002; Kesari et. al., 2007,Prabhu et al 2018). Methanolic leaf extract of *P.rajendranii* was investigated for in vitro antidiabetic activity with respect to

Table 3. Anti-inflammatory Activity of *Premna rajendranii*

SL.NO:	Concentration in (µg)	% of inhibition	
		Sample	Standard
1	25	6.88±0.02	9.79±0.01
2	50	33.88±0.01	13.54±0.02
3	100	34.98±0.03	44.86±0.05
4	200	36.08±0.1	87.78±0.01

Figure 2. Anti-inflammatory Activity of *Premna rajendranii*



inhibition of α-amylase. The concentrations of samples used for testing the inhibitory activity are 25, 50, 75 and 100 (µg). The inhibition activity of test samples showed optimum activity when compared to commercial drug at different concentrations. The maximum inhibition showed at 100 µg concentration was 79.3% and minimum inhibition showed at 25 µg concentration was 44.82%. IC50 value of sample (288.8) is higher than standard drug (246.7). Acarbose was used as standard drug (Table 2, Fig: 1). Protein denaturation is the major cause of Inflammation (Mizushima and Kobayashi, 1968) and it is a response to negative stimuli including injury, infection (Lumeng and Saltiel, 2011). Basically it was meant to destroy invading microorganisms, inactivate toxins, repairing and healing of injuries which might lead to life threatening hypersensitivity reactions. Inflammation is the major cause for several diseases including neurological, cardiovascular, intestinal, dental and renal disorders and also linked to diabetics, ageing, obesity, multiple sclerosis, pancreatitis, cancer (Sakat et al., 2010; Burns et al., 2001; Kuek et al., 2006; Grivennikov et al.,2010; Jenny,2012; Hoque et al.,2012; Marchant et al., Wyss- Coray et al.,2012).

Prescribed medicines for inflammation are mainly non steroidal anti- inflammatory drugs. These drugs have certain side effects causing gastric bleeding, ulceration, renal failure. Medicinal plants have a prolonged history of use as a remedy for inflammation (Insel, 1996; Rang et al., 2007). *Premna serratifolia* and *Premna tomentosa* have been reported to possess anti-inflammatory activity (Alam et al., 1993; Habtemariam et al., 2015). Methanolic leaf extract of *P.rajendranii* has shown high inhibition than standard at 50µl i.e. 33.88±0.01. The inhibition activity of other test samples (25µl, 100µl, 200µl) showed moderate activity when compared to standard drug. Diclofenac was used as the standard drug.

CONCLUSION

Our study demonstrates that the *P.rajendranii* acts as an antidiabetic and anti-inflammatory agent. The antidiabetic and anti-inflammatory activities may be due to the presence of various bioactive compounds especially flavonoids. More purification needs to be done and further research on *P.rajendranii* is necessary for isolating bioactive compounds and their mode of action.

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Evaluation of Antimicrobial Activity of Some Traditional Medicinal Plants

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ABSTRACT

Antimicrobial activity of aqueous and solvent extracts of *Adina cordifolia, Careya arborea, Careya arborea, Hiptage benghalensis* and *Lannea coromandelica* were tested against different bacteria (*Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Enterobacter aerogenes, Escherichia coli, Proteus mirabilis, Klebsiella pneumonia)* and fungi (*Fusarium oxysporum, Aspergillus niger, Curvularia lunata* and *Alternaria alternata)*. Out of the tested plants, *Cassia angustifolia* and *Adina cordifolia* were found effective in inhibiting the growth of test microorganisms. Ethanol extract of *C. angustifolia* exhibited the maximum antibacterial potential with 24.66 mm inhibition zone of against *S. aureus* followed by *E.coli* (22.62 mm), *B. subtilis* (21.63 mm), *E. aerogenes* (18.32 mm), *K. Pneumonia* (17.67 mm) and *B. cereus* with 15.00 mm of inhibition. Aqueous extract of *C. angustifolia* showed maximum growth inhibition against *B. cereus* with inhibition zone of 21.55 mm followed by *B. subtilis* (19.47 mm) and *E. coli* (16.32 mm). *A. cordifolia* was found effective against *B. cereus* (15.52 mm), *S. aureus* (10.49 mm) and *E.coli* (11.32 mm). Against test fungi, *C.angustifolia* was found potent against *F. oxysporum* with 60 % of mycelial inhibition followed 13.16 % of inhibition against A. alternate. Ethanol extract of *C. angustifolia* exhibited the strong antifungal potential against *C. lunata* with 22.93 mm zone of inhibition.

KEY WORDS: *ANTIBACTERIAL, ANTIFUNGAL, CASSIA ANGUSTIFOLIA, ADINA CORDIFOLIA, AQUEOUS EXTRACT, ETHANOL EXTRACT.*

ARTICLE INFORMATION

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INTRODUCTION

Mankind is blessed with medicinal plants, an expensive gift from nature. Medicinal plants have been used widely as alternative therapeutic tools for the prevention or treatment of many diseases (Cartea et al., 2010, Nagavani and Rao, 2010).The active principles present in the medicinal plants are unknowingly exploited in traditional methods for the treatment of numerous ailments (Adebajo et al., 1983).Researchers are gradually increasing their attention towards the natural products and looking for new active principles, which lead to develop better drugs against microbial infections(Lopez et al., 2001, Ibrahim, 1997, Philip et al., 2009).The antimicrobial compounds obtained from plants have more preventive and curative effect than the commercial antibiotics, hence antimicrobial compounds of plants have more clinical value than the commercial ones against the infectious strains of resistant microorganisms, (Jamshiya, 2017). About 800 plants have been in use in Ayurveda, whereas Siddha system of medicine makes use of 600 plants in different formulations and Unani and Amchi system together are practicing about 700 plants in their various preparations. Development of resistance by microbes against the available antibiotics has deepened the concerns for the identification of new and natural antibiotics.

Plants are considered as the rich sources of secondary metabolites and have been reported to have potential therapeutic values (Farnsworth, 1973, Farnsworth and Soejarto, 1985). Medicinal plants are gradually gaining more priority and interest from microbiologists, pharmacologists and drug developing institutions/industries of India and abroad for last few decades owing to their vast medicinal values. Antimicrobial activity of medicinal plants have been reported by several workers from in India and other parts of the world, (Bhawasar et al., 1965, Shah and Qadry, 1971, Radhakrishnan et al., 1976, Rastogi et al., 1990, Asolkar et al., 1992, Mehmood et al., 1997, Ahmad et al., 1998, Grainger Bisset, 2000, Behl and Srivastava, 2002, Sofowora et al., 2013 and Gaudillière, 2019). In the present investigation, five medicinal plants viz. *Adina cordifolia, Careyaarborea, Cassia angustifolia, Hiptage benghalensis* and *Lanneacoro mandelica* have been selected based on their traditional medicinal properties like chronic cough, jaundice, stomach ache, swelling in stomach, antiamoebic, anti inflammatory, antifertility, antityphoid, anticholera, anti anaemia, in treating burning sensation of the body, rheumatism, hyperdipsia, obesity, instrinsic hemorrhage, elephantiasis, inflammation, neuralgia, sprains and bruises as reported by many researchers, (de Saravia and Gaylarde, 1998, Arya et al., 2003, Costa et al., 2009, Sharma et al., 2009, Siddique et al., 2010a, Parveen and Shahzad, 2011). Hence the above plants were evaluated for their antibacterial and antifungal activity against both gram positive and gram negative bacteria and phyto-pathogenic fungi.

MATERIALS AND METHODS

Collection of plant material: Leaves of the selected plants were used for antimicrobial activity. The selected plant materials were collected from in and around region of Mysore. The samples were collected freshly in the air tight clean polythene bags and brought to laboratory, washed under running water to remove the dust and debris.

Microorganisms used in study: Bacteria: *Bacillus cereus*, *Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Enterobacter aerogenes, Escherichia coli, Proteus mirabilis, Klebsiella pneumonia*. Fungi: *Fusarium oxysporum*, *Aspergillus niger*, *Curvularia lunata* and *Alternaria alternata*.

Preparation of extracts: Aqueous extraction: Fresh and washed samples were used for the aqueous extraction and extraction was made at 1:1 volume. About 50 gm of sample was weighed and macerated with 50 ml of distilled water in the mortar and pestle. The macerate was kept overnight for exudation of bio chemicals and were filtered through double layered muslin cloth followed by Whatman No. 1 filter paper. Obtained aqueous extracts were stored at 4 °C for further use. Solvent extraction: The test plants which gave effective results against tested pathogens in aqueous extracts were subjected to solvent extraction with the help of Soxhlet apparatus. Shade dried samples of test plant leaf materials were coarse powdered. The plant materials were exhaustively extracted by solvents with an increasing polarity. The solvent series which are used in extraction based on increasing polarity are as follows; Petroleum ether, chloroform, ethyl acetate and ethanol. The extracted solvents were kept for evaporation and obtained extracts were stored at 5 °C for further use.

Antibacterial Assay: Agar well diffusion method: This method was carried out in-vitro with nutrient agar medium ((HiMedia, Mumbai). About 25 ml of sterilized, autoclaved nutrient agar medium was poured on to the sterilized petri-plates.in a laminar air flow to avoid contamination. The spore suspension was uniformly spread over the sterilized media in Petri-plates with the help of 'L' shaped glass rod. The 6 mm of wells were made on the media with the help of stainless steel cork borer. The wells were filled with the extracts of 100 µl each. Plates were incubated at 27 ± 2°C, three replicates were maintained for each treatment. Inhibition zone was observed after 24 h of inoculation and inhibition zone was measured with zone scale. The results were tabulated. Agar disc diffusion method: In this method, Sterilized disks were impregnated with extracts solutions of the substances to be examined, the test compound at a known concentration.

About 20 ml of sterilized, autoclaved Muller Hinton agar (Hi Media, Mumbai) media was poured into sterile petri-plates and were allowed to solidify inside laminar air flow. About 100 µl of bacterial cultures were spread over solidified, media using 'L' shaped glass rod. The test extracts of 100 µl (100 mg/ml) was impregnated onto a sterile disc. Each test plates contained a disc impregnated with extract, one disc as positive control (Gentamicin) and one disc served as negative control(solvent), all discs were placed at equidistant from each other. The inoculated plates were incubated at 37°C for 24 h. The experiment was carried out in triplicates and the zone of inhibition was recorded with zone scale.

Antifungal Assay: Poison Food Technique: Antifungal activity was carried out In-vitro at 10% of concentration. About 2 ml of 100% extract from the prepared stock solution (100%) was added to 20 ml of sterilized, autoclaved Potato Dextrose Agar (PDA) (HiMedia, Mumbai) media to bring up 10% of concentration, poisoned media in the plate were allowed to solidify. A 5 mm diameter of actively growing mycelium disc of the pathogen of 7days old culture was placed in the center of the Petri plates containing poisoned media plates. And plates were incubated at 27 °C. Three replicates were maintained for each treatment. Radial growth of mycelium was measured after seven days after inoculation. The percent inhibition of the fungus in treatments was calculated using following formula

$$\%\ of\ growth\ inhibition = \frac{dc - dt}{dt} \times 100 \quad (1)$$

Where;
dc= Average increase in mycelial growth in control, dt= Average increase in mycelial growth in treatment.

Agar disc diffusion method: In this method, sterilized disks impregnated with extracts of the substances to be examined, the test compound at a known concentration. About 20 ml of sterilized, autoclaved Potato Dextrose Agar (PDA) media was poured into sterile petri-plates and were allowed to solidify in laminar air flow. About 100 µl of fungal cultures were spread over solidified media using 'L' shaped glass rod. The test extracts of 100 µl (100 mg/ml) was impregnated onto a sterile disc. The inoculated plates were incubated at 27°C for 7 days. The experiments were carried out in triplicates and the zone of inhibition was recorded with zone scale.

Statistical analysis: All the experiments were carried out in triplicates and the data were subjected to one- way analysis of variance (ANOVA), followed by Tukey's post test at $P \leq 0.05$ level of significance using Graph pad prism 5 software.

RESULTS AND DISCUSSION

Antibacterial activity of aqueous extracts: In the present study five medicinal plants have been screened for antibacterial activity. Out of tested plants *Cassia angustifolia* has effectively inhibited growth of the pathogenic bacteria *B. cereus* with inhibition zone of 21.55 mm, *B. subtilis* (19.47 mm), *S. aureus* (15.76 mm), *E. coli* (16.32 mm) as compared to others followed by *Adina cordifolia B. cereus* (11.52 mm), *S. aureus* (10.49 mm) and *E. col*i (11.32 mm). The plant *Lanneacoromandelica* was effective against *B. subtilis* (11.66 mm) only. Whereas *Careyaarborea* and *Hiptagebenghalensis* has shown no activity on any bacterium (Table1, Fig. 1).

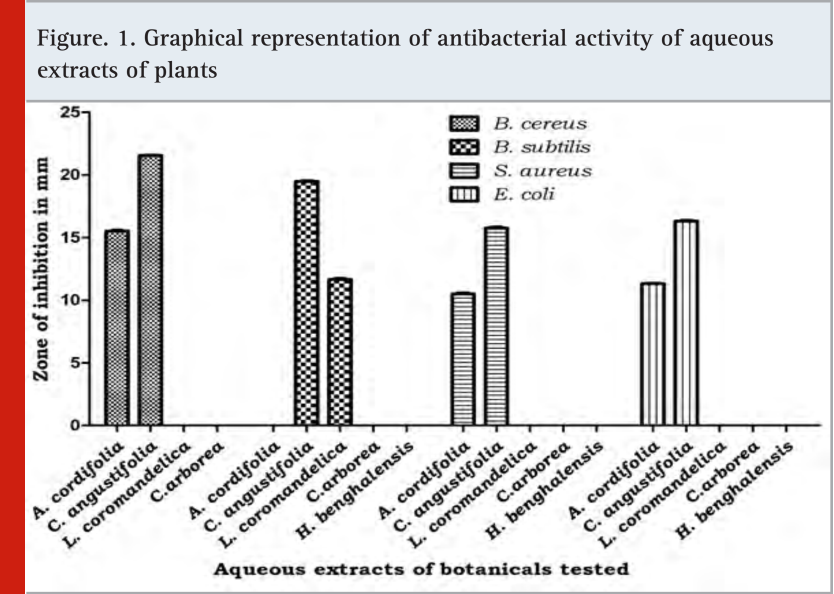
Antifungal activity of aqueous extracts: Antifungal activity was carried out by poisoned food technique at 10 %. The results revealed that *Cassia angustifolia* with percentage inhibition of 60% (*Fusarium oxysporum*), 13.16 % (Alternaria alternata), 11.20% (*Aspergillusniger*) and 09% (*Curvularialunata*) was the only plant which inhibited the growth of the tested fungi and whereas least inhibition was observed in

Table 1. Antibacterial activity of aqueous plant extracts against bacteria

Sl. No	Name of the plant	Parts used	Inhibition of Bacteria in mm Gram Positive Bacteria				Gram negative Bacteria			
			B. cereus	B. subtiltis	S. aureus	S. pyogenes	E. aerogenes	E . coli	P. mirabilis	K . pneumonia
1	*Adina cordifolia*	Leaves	11.52 ± 0.06*	0.00 ± 0.0	10.49 ± 0.08*	0.00 ± 0.0	0.00 ± 0.0	11.32 ± 0.04*	0.00 ± 0.0	0.00 ± 0.0
2	*Careyaarborea*	Leaves	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
3	*Cassia angustifolia*	Leaves	21.55 ± 0.04**	19.47 ± 0.06**	15.76 ± 0.07**	0.00 ± 0.0	0.00 ± 0.0	16.32 ± 0.05**	0.00 ± 0.0	0.00 ± 0.0
4	*Hiptagebenghalensis*	Leaves	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
5	*Lanneacoromandelica*	Leaves	0.00 ± 0.0	11.65 ± 0.04*	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0

Note: Asterisk marks *, **, *** indicate significance of values at P 6 0.05 level according to ANOVA, Tukey'spost test.

Figure. 1. Graphical representation of antibacterial activity of aqueous extracts of plants



Lanneacoromandelica with 7% inhibition of *Aspergillusniger*, *Hiptagebenghalens* is showed inhibition of *Fusarium oxysporumand Aspergillusniger* with inhibition percentage of 6% and 2.22% respectively. *Adina cordifolia* showed least inhibition against *Fusarium oxysporum* (2.22%) only. Whereas no inhibition was observed in the *Careyaarborea* for all the tested fungi (Table 2, Fig. 2).

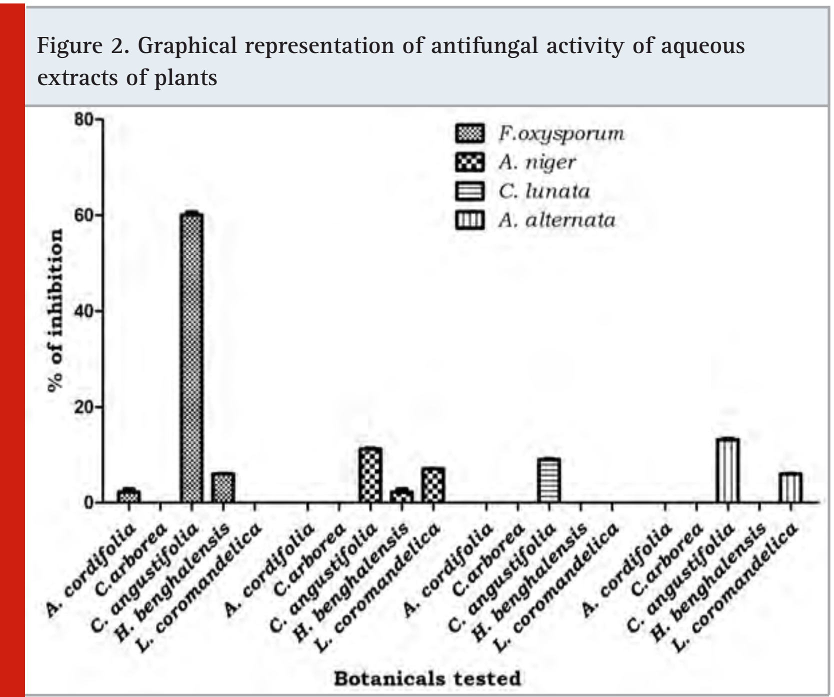
Antibacterial activity of solvent extracts: Based on the results obtained from the aqueous extracts two medicinal plants were selected for further activity. *Adina cordifolia* and *Cassia angustifolia* have been selected for further studies as they shown good activity against both bacteria and fungi as compared to others. These two plants were subjected to solvent extraction and the obtained extracts of solvents were examined for antibacterial activity. The results revealed that, both Petroleum ether and Chloroform of both plants shown no activity on all tested pathogenic bacteria. Ethyl acetate extract of *Adina cordifolia* effective only on S. aureus with inhibition zone of 15.31mm. Whereas ethanol extract of *Adina cordifolia* has shown inhibition of *S. aureus*, *B. Cereus* and *E. coli* with inhibition zone of 23.61 mm, 15.33 mm and 14.63 mm respectively. Ethyl acetate extract of *Cassia angustifolia* was effective on *S. aureus* and *E. coli* with inhibition

Table 2. Antifungal activity of aqueous plant extracts against fungi

Sl. No	Name of the plant	Parts used	Fusarium oxysporum	Percentage inhibition of Fungi Aspergill usniger	Curvulari alunata	Alternaria alternata
1	*Adina cordifolia*	Leaves	2.22 ± 0.64	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
2	*Careyaarborea*	Leaves	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
3	*Cassia angustifolia*	Leaves	60.00 ± 0.60***	11.20 ± 0.15	09.00±0.14	13.16 ± 0.16*
4	*Hiptagebenghalensis*	Leaves	06 ± 0.11	2.22 ± 0.64	0.00 ± 0.0	0.00 ± 0.0
5	*Lanneacoromandelica*	Leaves	0.00 ± 0.0	07.00 ± 0.08	0.00 ± 0.0	0.00 ± 0.0

Note: Asterisk marks *, **, *** indicate significance of values at P 6 0.05 level according to ANOVA, Tukey'spost test.

Figure 2. Graphical representation of antifungal activity of aqueous extracts of plants



zone of 22.31 mm and 13.65 mm respectively while ethanol extract shown effective inhibition of pathogenic bacteria *B.cereus, B. subtilis, S. aureus, E. aerogenes, E. coli,* and *K. pneumonia* with inhibition zone of 15 mm, 21.63 mm, 24.66 mm, 18.32 mm, 22.62 mm, 17.67 mm respectively (Table 3, Fig. 3).

Antifungal activity of solvent extracts: Results of antifungal activity revealed that solvent extracts of *Cassia angustifolia* was more effective than *Adina cordifolia*. Out of tested four solvent extractions ethyl acetate and ethanol extractions were effective against tested pathogens whereas petroleum ether and chloroform not shown any inhibition against any tested pathogens. Ethanol extract of *Cassia angustifolia* was more effective with inhibition zone of 14.33 mm *(Fusarium oxysporum*), 20.63 mm (*Aspergillusniger*), 22.93 mm (*Curvularialunata*) and 10.16 mm (*Alternaria alternata*) and ethyl acetate extract shown that 9.2 mm (*Fusarium oxysporum*), 15.43 mm

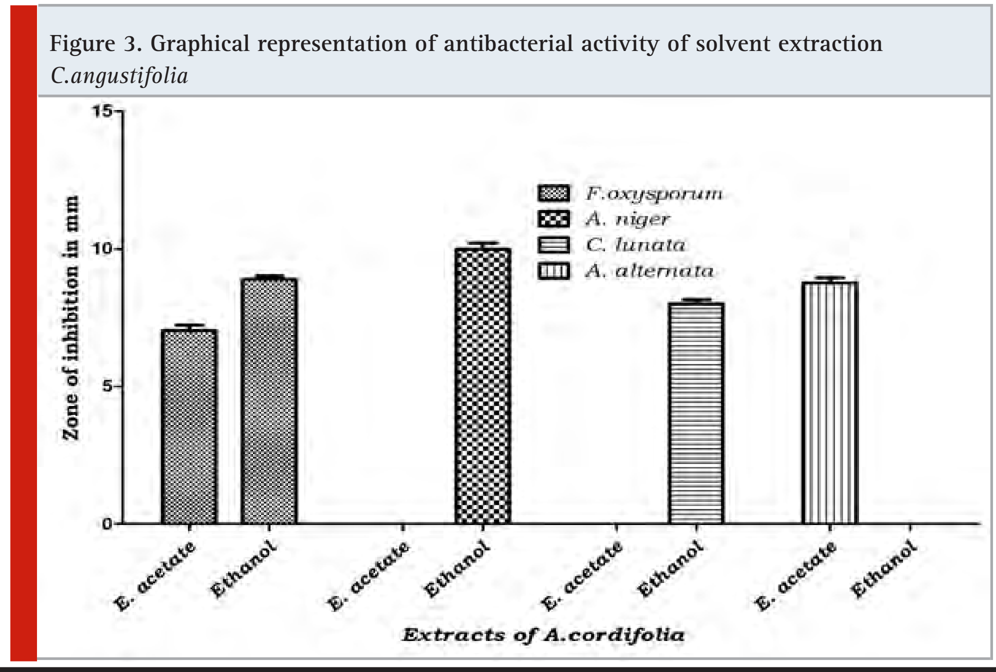
(Aspergillusniger), 17.53 mm (*Curvularialunata*) and 14.26 mm (*Alternaria alternata*). Ethanol extract of *Adina cordifolia* has shown inhibition zone of 8.9 mm (*Fusarium oxysporum*), 10.00 mm (*Aspergillusniger*) and 8.00 mm (*Curvularialunata*) whereas ethyl acetate extract of *Adina cordifolia* was effective only against *Fusarium oxysporum* and Alternaria alternata with inhibition zone of 7.03 mm and 8.76 mm respectively (Table 4, Fig. 4, 5). Natural products such as botanical extracts have bioactive principles which are produced as secondary metabolites; their main function is to

Table 3. Antibacterial activity of solvent plant extracts against bacteria

Sl. No	Name of the plant	Solvent extracts	Inhibition of Bacteria in mm Gram Positive Bacteria				Gram negative Bacteria			
			B. cereus	B. subtilis	S. aureus	S. pyogenes	E. aerogenes	E. coli	P. mirabilis	K. pneumonia
1	Adina	Petroleumether	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
	cordifolia	Chloroform	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
		Ethyl acetat	0.00 ± 0.0	0.00 ± 0.0	15.31± 0.02**	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
		Ethanol	15.33 ± 0.02**	0.00 ± 0.0	23.61 ± 0.02**	0.00 ± 0.0	0.00 ± 0.0	14.63 ± 0.05**	0.00 ± 0.0	0.00 ± 0.0
2	Cassia	Petroleumether	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
	angustifolia	Chloroform	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
		Ethyl acetate	0.00 ± 0.0	0.00 ± 0.0	22.31 ± 0.02**	0.00 ± 0.0	0.00 ± 0.0	13.65 ± 0.03*	0.00 ± 0.0	0.00 ± 0.0
		Ethanol	15 ± 0.17**	21.63 ± 0.05**	24.66 ± 0.06***	00. 00	18.32 ± 0.03**	22.62 ± 0.06**	0.00 ± 0.0	17.67 ± 0.07**
3	Gentamycin		28.60 ± 0.11***	24.29 ± 0.09***	27.26 ± 0.07***	21.60 ± 0.11**	25.59 ± 0.08***	26.53 ± 0.06***	22.21 ± 0.09**	28.21 ± 0.10***

Note: Asterisk marks *, **, *** indicate significance of values at P 6 0.05 level according to ANOVA, Tukey'spost test.

Figure 3. Graphical representation of antibacterial activity of solvent extraction *C.angustifolia*



protect host plants from pathogenic microbial invaders which affect the health of the host plants. As science and research has progressed, the scientific community has found to be aware of antimicrobial activity of botanicals and started to explore active principles of secondary metabolites from the plants. As a result of this huge number of plants around the world found to have antimicrobial properties, (Bhawasar et al., 1965, Behl and Srivastava, 2002, Chopra and Chopra, 1969, Iyengar, 1981, Mehmood et al., 1997, Grainger Bisset, 2000, Radhakrishnan et al., 1976, Shah and Qadry, 1971, Zafar, 1994 and Dahanukar and Hazra, 2009). In the present investigation in vitro study was carried out to evaluate the *aqueous* and solvent extractions five medicinal plants against pathogenic bacteria and fungi. In aqueous extracts significant result was observed in *C. angustifolia*, it has inhibited B. cereus with 21.55 mm of inhibition zone followed by *B. subtilis* (19.47 mm), *E. coli* (16.32 mm), *S. aureus* (15.76 mm), another plant *A. cordifolia* has shown inhibition of *B. Cereus* (15.52 mm), *S. aureus* (10.49 mm) and *E. coli* (11.32 mm) and *L. coromandelica* was effective only against *B. subtilis* (11.65 mm). Whereas, *C. arborea* and

Table 4. Antifungal activity of solvent plant extracts against fungi

Sl. No	Name of the plant	Solvent extracts	Fusarium oxysporum	Percentage inhibition of Fungi		
				Aspergillusniger	Curvularialunata	Alternaria alternata
		Petroleumether	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
01	*Adina*	Chloroform,	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
	cordifolia	Ethyl acetate	7.03 ± 0.20*	0.00 ± 0.0	0.00 ± 0.0	8.76 ±0.18*
		Ethanol	8.9 ± 0.11*	10.00 ± 0.18*	08 ± 0.15*	0.00 ± 0.0
		Petroleumether	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
2	*Cassia*	Chloroform,	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
	angustifolia	Ethyl acetate	9.2 ± 0.17*	15.43 ± 0.20**	17.53 ± 0.20**	14.26 ± 0.12**
		Ethanol	14.3 ± 0.20**	20.63 ± 0.17**	22.93 ± 0.14***	10.16 ± 0.17*

Note: Asterisk marks *, **, *** indicate significance of values at P 6 0.05 level according to ANOVA, Tukey'spost test.

Figure 4. Graphical representation of antifungal activity of solvent extraction A. cordifolia

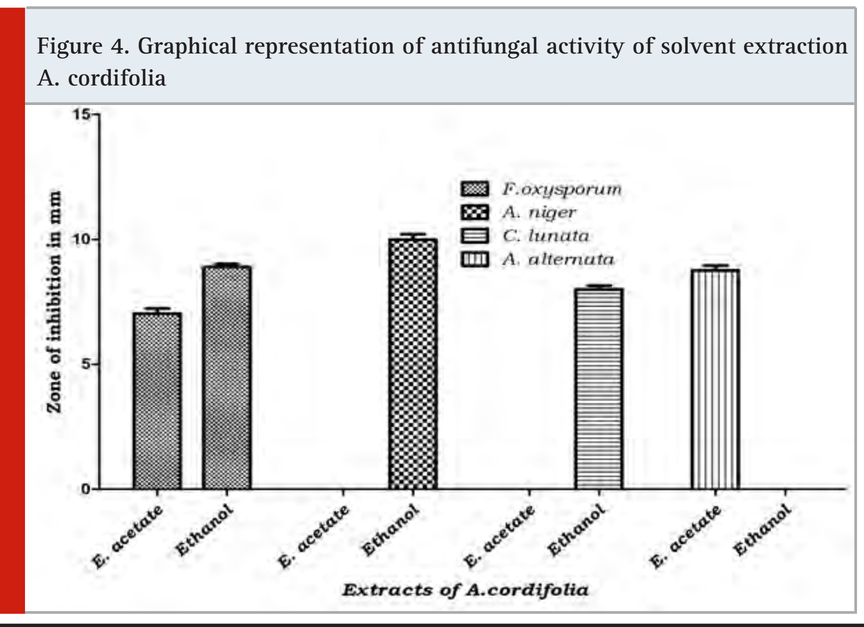
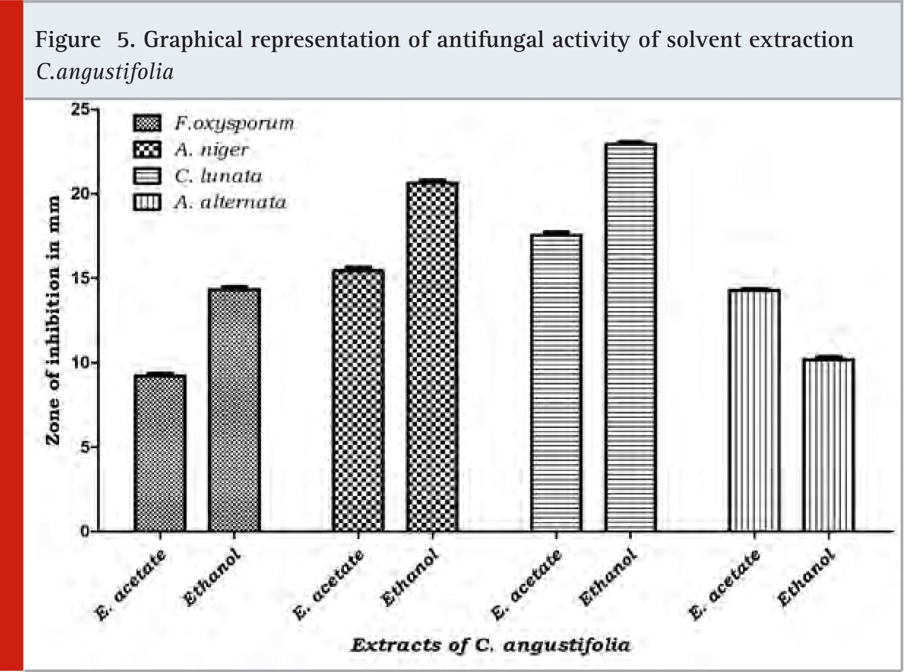


Figure 5. Graphical representation of antifungal activity of solvent extraction *C.angustifolia*



H. benghalensis not shown any activity against any bacteria. When aqueous extracts of plants tested against fungi only *C. angustifolia* shown effective results against *F. oxysporum* with inhibition percentage of 60%. Among solvent extracts ethyl acetate extraction of *A. cordifolia* was effective against *S. aureus* with inhibition zone of 15.31 mm and *C. angustifolia* against *S. aureus* and *E. coli*, with inhibition zone of 22.31 mm and 13.65 mm respectively. Ethanol extract of A. cordifolia was effective against *B. cereus, S.aureus* and *E. coli* with inhibition zone of 15.33 mm, 23.61 and 14.63 mm and C. angustifolia against S.aureus24.66 mm. whereas against A. niger, C. lunata and A. alternata not shown much inhibition, inhibition percentage are 11.20 %, 9.00% and 13.16% respectively. Some of the earlier reports corroborates with antibacterial and antifungal activity of extracts of the botanicals(Chen and Dai, 2012, de Saravia and Gaylarde, 1998, Goussous et al., 2013).

Also, other studies also confirms the antimicrobial capability of C. angustifolia of various extracts against S. mercescens, A. junni, E. cloacae, Aspergillus niger, Aspergillus terreus, Aspergillus flavus, and *Aspergillus fumigates*, (Al-Marzoqi et al., 2016, Ahmed et al., 2016). Based on the results of the aqueous extracts the plants which given significant results, two plants were selected for further solvent extraction, the results revealed that only ethyl acetate and ethanol solvent extraction showed activity where as others not shown any activity on any tested pathogens either on bacteria or fungi. Ethyl acetate extraction of *A. cordifolia* was only effective against S.aureus with inhibition zone of 15.31 mm whereas ethyl acetate extraction of *C. angustifolia* was effective against *S.aureus* and *E. coli*, with inhibition zone of 22.31 mm and 13.65 mm. Ethanol extract of *A. cordifolia* was effective against *B.cereus, S.aureus* and *E. coli* with inhibition zone of 15.33 mm, 23.61 and 14.63 mm whereas ethanol extract of C. angustifolia was effective against maximum tested bacteria except *S. pyogenes* and *P. mirabilis* and shown maximum inhibition zone of 24.66 mm against *S.aureus* and 22.62 mm, 21.63 mm 18.32 mm, 17.67 mm and 15.00 mm against *E. coli*, *B. subtilis*, *E. aerogenes, K. pneumonia* and *B. cereus* respectively. There are earlier reports that solvent extracts of botanicals has antibacterial activity, (Abas et al., 2006,

Shan et al., 2007, Kang et al., 2011, Hosamath, 2011, Parveen and Shahzad, 2011,). In the antifungal activity of solvent extraction against ethyl acetate and ethanol extractions shown activity whereas petroleum ether and chloroform not shown any activity against all tested phytopathogenic fungi. Ethyl acetate extraction of *A. cordifolia* shown inhibition of *F. oxysporum* and *A. alternate* with inhibition zone of 7.03 mm

and 8.76 mm whereas ethyl acetate extraction of *C. angustifolia* was effective against all the tested fungi with inhibition zone of 17.53 mm (*C. lunata*), 14.26 mm *(A. alternata)* and 9.2 mm *(F. oxysporum)*. Ethanol extraction of both plants shown effective inhibition of the phytopathogenic fungi. Ethanol extraction of *A. cordifolia* was effective against *F. oxysporum* with inhibition zone of 8.9 mm, A. niger (10.00 mm) and *C. lunata* (8.00 mm). Ethanol extract of *C angustifolia* has shown more effective than any other tested solvents with inhibition zone of 22.93 mm against *C. lunata, A. niger* (20.63 mm), *F. oxysporum* (14.3 mm) and *A. alternata* (10.16 mm). The present investigation came out with medicinal plant *C. angustifolia* which was effective against both tested bacteria and fungi in both aqueous extracts and solvent extractions. There were many reports of medicinal values of *C. angustifolia* from various researchers. The plant has been in use as traditional medicine, (Wu et al., 2009), the plant is also used in the treatment of amoebic dysentery, typhoid, cholera, anemia(Siddique et al., 2010b, Wu et al., 2009, Parveen and Shahzad, 2011).

CONCLUSION

The study confirms the antimicrobial potential of leaf extracts of *Adina cordifolia*, *Careya arborea*, *Cassia angustifolia*, *Hiptage benghalensis* and *Lannea coromandelica* and thereby confirms their traditional medicinal use. Bioprospection of ethnomedicinal plants paves a way for the identification of natural and novel drugs. Further identification and characterization of biologically actives principles needs to be done which may lead to the discovery of potent antimicrobial agents.

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Authors' Contribution: Kh. Tahamtan and M.S Sharada developed ideas and drafted the manuscript. Dr.M.S Sharada provided the required facilities. Kh. Tahamtan conducted the experiments. Kh .Tahamtan and Dr. M.S Sharad had contributed to the design and analysis of data. Authors contributed to revise the manuscript and approved the final version for publication.

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Characterization of Siderophore Producing *Pseudomonas Sp* for its Plant Growth Promoting Properties

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ABSTRACT

Siderophores are small molecules and important iron chelators produced by microbes like bacteria,algae, and fungi. This study was undertaken to determine the efficiency of rhizobacterial isolates to produce different types of siderophores that enhance the plant growth promoting substances like Indole acetic acid, HCN production, Phosphate solubilisation, Ammonia production etc. The isolates from the rhizosphere soil region are characterized morphologically and biochemically and identified as Pseudomonas sp. The strains were assessed for their siderophore production by Chrome azurol S assay. The results indicates that isolates were able to produce hydroxmate and carboxylate type of siderophore and these isolates have studied for their PGPR activity. The results shows that the isolates have a potential on plant growth promotion and they enhance the plant growth efficiently.

KEY WORDS: CHROME AZUROL S ASSAY, INDOLE ACETIC ACID, PLANT GROWTH PROMOTING RHIZOBACTERIA, SIDEROPHORES

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INTRODUCTION

Microbes are diverse in nature which is present in all surrounding areas of earth. In terms of sheer number and mass it is estimated that microbes contain 50 % of the biological carbon and 90 % of the biological nitrogen on earth. Soil microbial communities appear to be more diverse than those found in most fresh water and marine environments. The vicinity of plant roots are surrounded by rhizosphere which is an extremely important and active area for root activity and metabolism. (Agrawal et.al., 2015). The Pseudomonas sp encompasses arguably the most diverse and ecologically significant group of bacteria on the planet. Fluorescent Pseudomonas is gram negative, rod shaped, chemoheterotrophic bacteria with polar flagella and are characterized by the yellow green pigments (Sullivan et.al., 1992).

Fluorescent Pseudomonas is well recognized as plant growth promoting rhizobaceria, phosphate solubilizers and also act as biocontrol agents against many soil born plant pathogens, (Battu et.al .,2009). Three different characteristics have been determined in order to classify the strains as plant growth promoting rhizobacteria. These characteristics are 1.ability to produce auxin,2. Sidrophores 3. The solubilisation of phosphates presents in the medium (Cardenas et al., 2019) Plant Growth Promoting Rhizobacteria (PGPR), which enhances plant growth and increase crop yield via secretion of various plant growth promoting substances as well as biofertilizers. PGPR's exhibit antagonistic effects to soil-borne pathogens or induce the systemic resistance against pathogens in the entire plant lifespan, (Swamy et at ., 2019).

Siderophores are relatively low molecular weight, ferric ion specific chelating agents synthesized by bacteria, actinomycetes, fungi and certain algae growing under low ionic stress(Kannahi et.al ., 2014). The iron ligation groups have been tentatively classified into three main chemical types: hydroxamate, catecholate and hydroxycarboxylates. The type of siderophore synthesized by bacteria depends on the amount and accessibility of nutrients and it may differ in culture rich conditions as compared to natural habitat. (Maheshwari, et al.,(2019). Pseudomonas fluorescence and Pseudomonas putida produce siderophores of two general types, pyochelin and pyoverdin(Dava et.al.,2000). Pyoverdin are water soluble pigments that fluoresce under ultra violet light (Budzikiewicz et.al., 1993). Siderophores produced by rhizosphere inhabitants has been studied well and it has been reported that ability to produce siderophores not only improve rhizosphere colonization of producer strain but also play an important role in iron nutrition of plant.

MATERIAL AND METHODS

Sample collection

Rhizosphere soil sample was collected from Palladam region, Tirupur district, Tamil Nadu, South India. The collected soil sample was brought to the laboratory in a sterile ziplock bag aseptically and maintained at the laboratory for further study. Isolation of bacteria: 0.1 ml of serially diluted sample was taken from 10-4 to 10-7 dilutions, was spreaded on King's B medium plates and incubated at 28^{0}C for 48 h. After incubation the plates were exposed to UV light at 365 nm for few seconds and the colonies exhibiting the fluorescence were picked up and purified on King's B medium plates.

Characterisation and identification: Phenotypic characterisation was carried out by subjecting the bacterial isolates to morphological (colony morphology), microscopical (grams staining), motility, and biochemical tests (utilisation of different carbon sources and enzyme activity) following standard procedure as per Bergey's manual of systematic bacteriology. Production And Detection Of Sidersophores: CAS Agar Plate Technique (Agrawal et.al.,2015: Siderophore production by all the isolates were tested by chrome azurol dye method. Freshly grown bacterial cultures were inoculated into CAS agar plate and incubated at 37°C for 24 -48 hours. After incubation appearance of an orange zone against the dark blue background indicates the siderophore production.

Tetrazolium Salt Test to Detect Hydroxamate Type of Siderophore (Syamala et al 2017): To a

pinch of tetrazolium salt, 1-2 drops of 2N NaOH and 0.1ml of test culture supernatant were added. Instant appearance of deep red colour indicated hydroxamate nature of siderophores. Vogel's Chemical Test To Detect Carboxylate Type Of Siderophore: To 3 drops of 2N NaOH , 1 drop of phenolphthalein and then water was added until light pink colour was developed. Disappearance of pink colour on addition of test sample, indicate carboxylate nature of siderophores.

Detection of Plant Growth Promoting Substances

Indole Acitic Acid Production:(Agrawal et.al.,2015): Isolates were inoculated in King's B medium supplemented with 0.1mg/ml tryptophan and incubated at 37^{0}C for 4 days. The cells were removed by centrifugation at 10,000 rpm for 15 minutes. 1ml of the cell free supernatant was transferred to a fresh tube to which 50µl of 10mm orthophosphoric acid and 2ml of Salkowski's reagent was added. The tubes were observed for the presence or absence of pink colour.

Phosphate Solubilization: (Agrawal et.al.,2015): The Pikovskaya's medium was prepared and sterilized at 121oC for 15 minutes at 15lbs pressure. It was poured onto sterile petriplates, inoculated and incubated with the bacterial isolates at 37^{0}C for 4-5 days. The plates were examined for the presence or absence of clear zone surrounding each test isolates.

Figure 1. Fluorescence Colonies on Kings B Agar

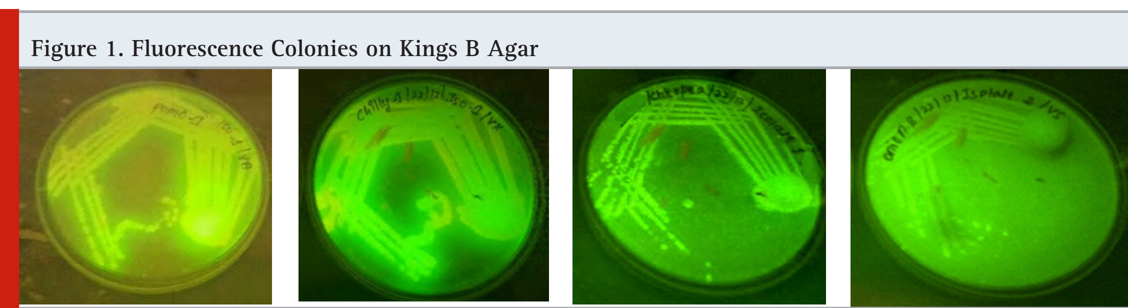

Table 1. Morphological identification of Pseudomonas strains Ps_1 – Ps_9

S.no	Sample	Colony morphology	Grams nature	Motility	Pigment production	Fluorescence under UV
1	Pomegranate Ps_1	Opaque round colony	Gram Negative, rod	Motile	Yellowish colony	Yellow Fluorescence colony
	Pomegranate Ps_2	Round colony	Gram Negative, rod	Motile	Yellowish colony	Yellow Fluorescence colony
2	Chilly Ps_3	Transparent colony	Gram Negative, rod	Motile	Yellowish green	Fluorescence colony
	Chilly Ps_4	Opaque colony	Gram Negative, rod	Motile	Yellowish green	Fluorescence colony
3	Chickpea Ps_5	Dome shaped colony	Gram Negative, rod	Motile	Yellow	Yellow fluorescence colony
	Chickpea Ps_6	Small pointed colonies	Gram Negative, rod	Motile	Yellowish Green	Yellow Fluorescence
	Chickpea Ps_7	Small dome shaped colonies	Gram Negative, rod	Motile	Yellowish green	Greenish Fluorescence
4	Onion Ps_8	Opaque colony	Gram Negative, rod	Motile	Yellowish Green	Greenish Fluorescence
	Onion Ps_9	Transparent colony	Gram Negative, rod	Motile	Yellowish Green	Greenish Fluorescence

HCN Production:(Syamala et al 2017): All the isolates were screened for the production of hydrogen cyanide .King's B medium was mixed with 4.4g of glycine and the isolates were streaked onto the agar plate. Whatmann filter paper soaked in the solution of 2% sodium carbonate and 0.5% picric acid solution was placed on streak plate's upper lid and the plates were sealed with paraffin. The plates incubated at 37oC for 4-5 days. The change in the colour from yellow to orange red indicates the HCN production.

Protease Production: (Apastamph et.al 2014):The active bacterial cultures were spot inoculated on caseincontaining sterile media plate and incubated at 30 ^{0}C for 48 hrs and observed for zone of clearance.

Cellulose Production: Apastamph et.al 2014): The Czapek mineral salt media was prepared and sterilized at 121^{0}C for 15 minutes at 15 lbs pressure. It was poured on a sterile petriplates, inoculated and incubated with bacterial isolates at 37oC for 24 hours. The plates were examined for the presence or absence of clear zone surrounding each test isolates on exposure to 1% Congo red and 1M sodium chloride.

Nitrogen Content: (Kaushal et al., 2013): Nitrate broth was inoculated with bacterial cultures and incubated at 30^{0}C for 48 hrs3 drops of reagent and 1drop of sulphuric acid were added in a petriplate and 1 drop of culture was added to it. Blue colour appeared indicates nitrite is produced. Zinc dust was added, if no blue colour appeared indicates nitrate was reduced to nitrite.

RESULTS AND DISCUSSION

The organisms were isolated from rhizosphere soil, after 24 hrs the colonies were appeared as crowded, it was then purified on kings B agar plate and observed under UV light at 365 nm. Strains were identified based on its cultural, morphological, microscopic and motility tests. The colonies that can able to produce yellow green colour pigment have been selected for further analysis (Battu et al 2009). The isolates were further maintained on nutrient agar slants and used for furthers studies. (Table 1) (Figure 1) . With the help of biochemical characteristics, as per bergys manual of determinative bacteriology the colonies were named as Pseudomonas Ps 1 to Ps9 respectively. Results showed that the strains were positive for citrate, catalase positive,Urease positive, liquefy gelatinase, and produce H2S. The

Table 2. Physiological and Biochemical properties of the selected bacterial isolates

	TEST	Ps_1	Ps_2	Ps_3	Ps_4	Ps_5	Ps_6	Ps_7	Ps_8	Ps_9
1	Indole production test	-	-	-	-	-	-	-	-	-
2	Methyl red test	-	-	-	-	-	-	-	-	-
3	Voges proskauer test	-	-	-	-	-	-	-	-	-
4	Citrate utilization test	+	+	+	+	+	+	+	+	+
5	Oxidase test	-	-	-	-	+	+	+	+	+
6	Catalase test	+	+	+	+	+	+	+	+	+
7	Gelatin hydrolysis	+	+	+	+	+	+	+	+	+
8	H_2S production test	+	+	+	+	+	+	+	+	+
9	Urease test	+	+	+	+	+	+	+	+	+
10	Triple sugar iron test	AB/AS	AB/AS	AB/AS	AB/AS	AB/AS	AB/AS	AB/AS	AB/AS	AB/AS
	Carbohydrate fermentation tes									
1	Glucose	+	+	+	+	+	+	+	+	+
2	Lactose	-	-	-	-	-	-	-	-	-
3	Mannitol	+	+	+	+	+	+	+	+	+
4	Sucrose	+	+	+	+	+	+	+	+	+

Note : + -positive,- -negative ,AB/AS Acid butt/ Acid slant

strains were able to produce acid butt and alkaline slant.It was also observed that it is positive for oxidase Ps 5 –Ps9 (Holt et al 1994). All the isolates are positive for urease production. The stains can able to utilize various type of carbohydrates like glucose,sucrose and mannitol and it shows negative results for lactose utilization.(Table 2). Siderophore are iron specific compounds which are synthesized and secreted under iron stress condition.(Budizikiewic 1993) Pseudomonas sp have been known for their siderophore production for many years and therefore many reports on the isolation and characterization of their siderophores have been published (Dava et al.,2000). Siderophores produced by Pseudomonas sp., have been employed efficiently as biocontrol agent against certain soil born plant pathogens (Fekadualemu 2013). Appearance of orange zone after 48 hrs of incubation indicates that Ps 1 to Ps 9 were able to produce siderophore in Chrome Azural ager medium(Belkar Y.K. et al., 2012).

Tetrazolium and neilands spectrophotometric assay is used for hrdroxamate type of siderophore ,Arnows assay is used for catecholate type of siderophore,Vogals assay for carboxylate type of sideophore .(Table 3). Instant appearance of deep red colour in tetrozolium test indicate the presence of hydroxamate nature of siderophore , disappearance of pink color on the addition of phenolphthalein indicates the carboxylate type of siderophores.(Syamala et al 2017). Strains Ps 1-2 and Ps 5-7 were able to produce hydroxamate and carboxylate type of siderophores is used for further analysis. Siderophores have been classified based on their main chelating groups. Generally they are categorized into two groups. (Payne 1994) . Siderophores in the rhizosphere region of the plant provide iron nutrition,serve us a first defence against root invading parasites and helps in removing toxic metals from the plants. Pseudomonas sp can enhance the plant growth by producing pyoveridine siderophores. (Joseph et al 2007)

Table 3. Detection and types of Siderophores

s.no	Strain no.	Types of siderophores	
		Hydroxemate	Carboxylate
1	Ps_1	+	+
2	Ps_2	+	+
3	Ps_3	-	-
4	Ps_4	-	-
5	Ps_5	+	+
6	Ps_6	+	+
7	Ps_7	+	+
8	Ps_8	-	-
9	Ps_9	-	-

Plant growth promoting properties of the selected Pseudomonas strains: The product of microbial metabolism that are released into the soil influence the growth of plant. Interaction between plant and microbes is well known for beneficial effect and such free living bacteria isolated from the rhizosphere of plants are known as plant growth promoting rhizobacteria (Kloepper et al.,1980). Pseudomonas sp showed the positive reaction towards IAA, (Agrawal et.al 2015). The presence of tryptophan in the medium significantly enhanced the indole acetic acid production. Indole acetic acid is the main auxin having influence on cell enlargement,division,tissue differentiation and response to light and gravity (Josip colo et al.,2014). Diverse bacterial species has the ability to produce IAA, (Reetha et al ., 2014). The improved plant growth is due to the ability of the organism to produce phytohormones such as indole acetic acid,gibberilic acid, cytokinins etc.

IAA producing bacteria may be a efficient biofertilizer inoculants to promote plant growth and protecting the medicinal plants for future generations (Glick 1995). The rhizosphere microorganism especially fluorescent pseudomonas have exceptional ability to promote the growth of the host plant by various mechanism to suppress plant disease including production of powerful siderophores, (Sullivan et al., 1992) Furthermore siderophores are able to reduce the oxidative stress by inhibiting the free radicle formation and can inhibit the IAA destruction. (Table 4) The isolates were positive for protease production, cellulose production,nitrate reduction (Apastamph et al., 2014). Qualitative analysis showed that all the bacterial isolates produce IAA,ammonia,siderophore etc (Anitha et al.,2013). The selected isolates were found to be positive for conversion of ammonia into nitrate

Table 4. Plant growth promoting properties of the seleced *Pseudomonas strains*.

S.NO	TEST	Ps_1	Ps_2	Ps_5	Ps_6	Ps_7
1	IAA production	+	+	+	+	+
2	HCN production	-	-	+	+	+
3	Ammonia production	+	+	+	+	+
4	Phosphate solubilization	+	+	+	+	+
5	Nitrate reduction	+	+	+	+	+
6	Cellulose	+	+	+	+	+
7	Protease	+	+	+	+	+

that plants can absorb and use it, (Ahmed et al.,2008).

Phosphorous is an essential nutrient for plant growth but often limiting due to its low solubility and fixation in the soil (Deshwal et al., 2013). The ability of the microorganisms to convert insoluble phosphorus to soluble form is an important trait in PGPR for increasing plant yield (Rodriquez et al., 2006) .Strains has shown to be positive for tricalcium phosphate solubilizers .Fluorescent pseudomonas have also been reported as phospahate solubilizers due to the excretion of organic acids (Banu et al.,2004). The exact mechanism by which PGPR stimulate the plant growth is not clearly known, although the mechanisms such as activation of phosphate solubilisation and promotion of mineral nutrient uptake are usually believed to be involved in plant growth promotion. The HCN production is found to be a common trait of Pseudomonas sp and Bacillus in the rhizospheric soil and plant root nodules, (Ahmed et al., 2008).Rhizosphere colonizing Fluorescent Pseudomonas significantly increased growth and yield of crops.

CONCLUSION

The results suggested that pigment producing Pseudomons sp present in the rhizosphere soil region. All the strains can able to produce sideophores ,among these the strains Ps 1-2 to Ps 5-7 that can able to produce hydroxmate and carboxylate type of siderophores was selected and analysed for their plant growth promoting properties. These results shows that siderophore producing Pseudomonas sp has been an efficient plant growth promoters and control the plant pathogen by acting as a first defence. However, to ascertain the effectivity of PGP traits on growth and productivity of crops, through phytohormone production and control against harmful microbes, needs to be evaluated in further studies.

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Novel Method for the Segregation of Heart Sounds from Lung Sounds to Extrapolate the Breathing Syndrome

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ABSTRACT

Heart sound (HS) impede with lung sounds in a manner that obstructs the prospective of respiratory sound analysis in terms of diagnosis of respiratory disease. Lung sound (LS) signal measurements are taken to aid in the diagnosis of various diseases. Their elucidation is difficult however due to the presence of hindrance generated by the heart. Sound samples are recorded using Electronics stethoscope consist of both HS and LS where LS is separated from the HS for Asthma analysis at the crucial stage of the disorder. This proposal involves two main segments namely Respiratory Sound separation and analysis of the resultant LS. The separation of LS involves detection, annulment, localization and alignment of HS segments, modeling and prediction of LS. The recorded sound is given as output to the HS detection block and the segments of HS are detected using Multi resolution Analysis (MRA).

KEY WORDS: HEART SOUND, LUNG SOUND, ADAPTIVE FILTERS, LABVIEW, ELECTOROCULOGRAM (EOG) ,CURSOR CONTROL, HUMAN COMPUTER INTERACTION (HCI), LABVIEW.

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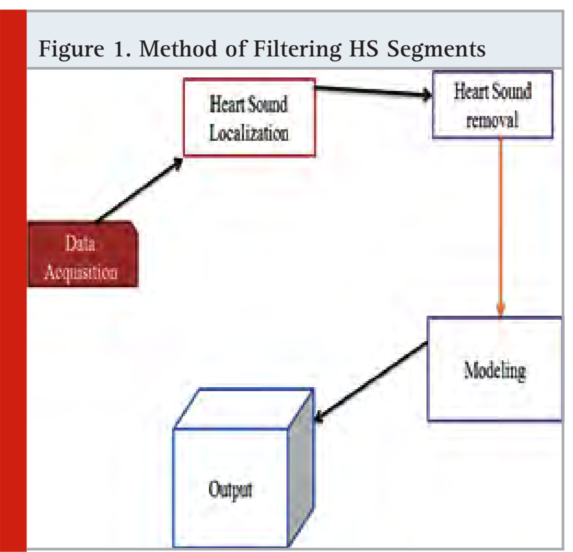
INTRODUCTION

Consistently 100 to 150 million individuals around the globe experience the ill effects of asthma and this number is expanding. Every year 1, 80,000 individuals kick the bucket because of asthma around the world. In India 15-20 million individuals have been evaluated to be influenced by Asthma.(Babu, 2019). Auscultation is a standout amongst the most vital non-intrusive and straightforward analytic devices for identifying issue in the respiratory tract like lung illnesses (AyeshaRumana.2014). It is characterized as the demonstration of listening for sounds inside the body, chiefly for learning the state of the lungs, heart and different organs (Palanivel Rajan, 2014). Sicknesses, for example, asthma, and tuberculosis can be related to this technique through the examination of lung and tracheal sounds.

Research on the diagnosis of respiratory pulmonary conditions like bronchitis, sleep apnea, asthma has established the utility of the stethoscope's acoustic signal in common day to day practice. However, despite their effectiveness, these instruments only provide a limited and subjective perception of the respiratory sounds. The drawbacks of using stethoscopes and listening to the sounds using the human (ear area) their inability to provide an objective study of the respiratory sounds detected, their lack of sufficient sensitivity and (c) the existence of the

Figure 1. Method of Filtering HS Segments



imperfect system of nomenclature (Thato, 2008).In the last few decades, improvements in electronic recording and the development of computer-based methods have made quantitative studies of lung and tracheal sound signals possible as well as overcome many limitations of human ear subjective auscultation. Modern digital processing techniques, along with advancements in computer analysis, have become an established research method for the investigation of respiratory sounds (Palanivel Rajan 2019). Automated respiratory sound analysis can quantify changes in lung sounds, de-noise the signals of interest from any artifacts and nosiness, store records of the measurements made, and produce graphical representations of characteristic features of the respiratory sounds to help with the diagnosis and treatment of patients suffering from various lung diseases (Xie, 2019). In view of the fact that lung sounds have quite low frequency and low intensity, interfering sounds (i.e., heart sounds) from the lung sounds prior to any diagnostic analysis. Efficacy of stethoscope plays an important role in the diagnosis of respiratory pulmonary conditions like bronchitis, sleeapnea, asthma in common day to day practice. Asthma is a persistent lung disease, a very common respiratory condition also known as reactive air way disease.

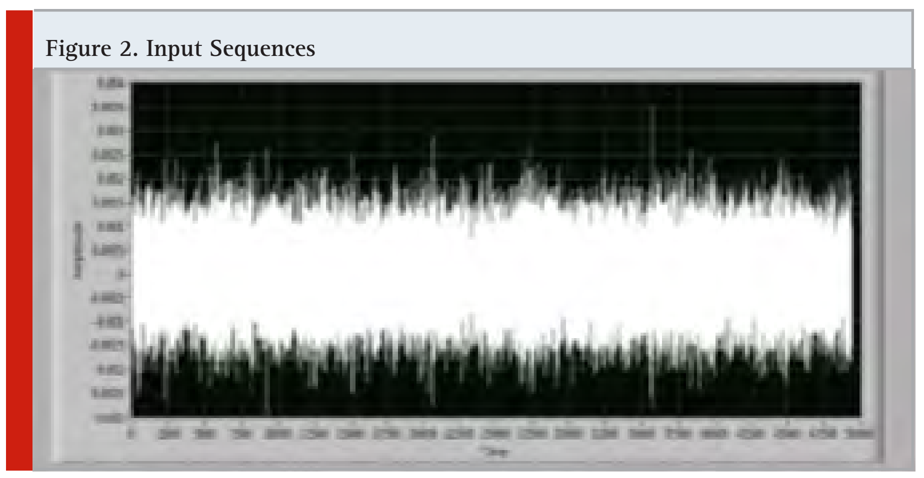
Asthma causes an inflammation and constriction of bronchial walls leading to a series of complications in breathing. Asthma can be diagnosed by the presence of the signs and symptoms. Confirmed diagnosis is performed using pulmonary function tests, chest x-rays and blood tests.The authors offered some diagnostic methods to be had for asthma by assessing the breathing patterns for asthmatic patient and also paying attention the need for the computational analysis of breathing pattern, alternate solution to diagnose asthma at the primary stage (Huang, 2019). In the past decades developments of electronic recording using computer-based method have made recent researches in the area of lung and tracheal sound signal analysis. Recent advancements in computer analysis and modern digital signal processing techniques have established more research methodologies in the investigation of respiratory sound. Biological signals are often noisy and

non stationary. These factors tremendously complicate analysis of bio-signals. Respiratory sounds present on invasive measures of lung air way conditions. However, features of lung sounds (LS) may be contaminated by heart sounds (HS) because lung and heart sounds overlap in terms of time domain and spectral content .Generally, all LS originate from air ways during inspiration expiration cycles .The LS propagating through lung tissues in the par enchyma can be heard over the chest wall using a sound-transducer.

The tissue acts as a frequency filter like structure whose characteristics vary according to pathological and indeed physiological changes, (Palanivel Rajan, 2017). Auscultation is one of the most important non-invasive and simple diagnostic tools for detecting disorders in the respiratory tract like lung diseases. However, despite their effectiveness, these instruments only provide a limited and subjective perception of the respiratory sounds. The drawbacks of using stethoscopes and listening to the sounds are using the human ear area, and their inability to provide an objective study of the detected respiratory sounds. They lack sufficient sensitivity and the existence of imperfect system of nomenclature. (Vijayprasath, 2015). Besides the fact that normal and abnormal lung sounds are mixed in the air ways and therefore pose a problem of classification of respiratory diseases, semi-periodic HS from heart beat activity invariably interferes with the LS and therefore masks or inhibits clinical interpretation of LS particularly over low frequency components.(Gnitecki, 2007) The main frequency components of HS are in the range 20-100Hz. This is the range in which LS have major components .Therefore, since HS and LS overlap in frequency and are somewhat non-stationary, the major problem being faced in separating HS from LS is doing so without tempering with the main characteristic features of the LS. Generally, LS are produced during inspiration and expiration cycles, and are found in the frequency range 20 - 1200 Hz. There are two types of lung sound, namely, normal and abnormal lung sounds. Normal breath sounds can be categorized into three classes: bronchial, bronchi vesicular, and vesicular sounds. Each class of sounds is detected during auscultation according to the characteristics described in (Gao, 2010).HSs are clearly audible in lung sounds recorded on the anterior chest and may be heard to a less ere tent in lung sounds recorded over posterior lung lobes.

High-pass filtering of lung-sound recordings to reduce heart sounds would remove significant components of lung sounds. Filtering techniques are categorized as linear adaptive filters and filters employing time-frequency based methods. Several filtering schemes are outlined within these two categories. In (Gnitecki, 2003), a recursive least squares (RLS)based adaptive noise cancellation (ANC) filtering technique is proposed to separate or reduce the HS from LS. Here, a band pass filtered version of the recorded HS was used as the reference signal. Time frequency (TF) filtering techniques have also been proposed for HS reduction in LS (Palanivel Rajan, 2013).

Figure 2. Input Sequences



Methods of heart sound localization are indicated in conjunction with the studies of heart-sound cancellation. Same researchers confirm that the adaptive filter is more effective in reducing noise from time series data than linear filters, wavelet shrinkage, and chaos based noise reduction schemes. The simplest method to reduce HS effects is to apply a high pass filter with cut-off frequency from 50-150Hz (Schuttler, 1997). More complex methods to reduce HS from breath sound recordings have been described in the literature as adaptive filtering techniques (Potdar, 2012), wavelet denoising, and combination of HS localization and removal and LS prediction. Respiratory sounds present non invasive measures of lung air way conditions (Mayorga, 2019).

However, features of lung sounds may be contaminated by heart sounds because lung and heart sounds overlap in terms of time domain and spectral content. Heart sounds are clearly audible in lung sounds recorded on the anterior chest and may be heard to a lesser extent in lung sounds recorded over posterior lung lobes. Lung sounds are produced by vertical and turbulent flow within lung air ways during inspiration and expiration of air [. Lung sounds recorded on the chest wall represent not only generated sound in lung airways brutal so the effects of thoracic tissues and sound sensor characteristics on sound transmitted from the lungs to a data acquisition system . Lung sounds exhibit a Power Spectral Density (PSD) that is broad band with power decreasing as frequency increases (Hadjileontiadis, 1998).

The logarithm of amplitude and the logarithm of frequency are approximately linearly related in healthy subjects provided that the signals do not contain adventitious sounds. As the flow in lung airways increases, sound intensity increases and several mathematical relations between lung sounds and airflow have been proposed. It is important to note that inspiratory and expiratory lung sounds differ in terms of both amplitude and frequency range. At comparable flows, aspiratory lung sounds will exhibit greater intensity than expiratory sounds. (Mohanapriya, 2013). Heart sounds are produced by the flow of blood into and out of the heart and by the movement of structures involved in the control of this flow. The first heart sound results when blood is pumped from the heart to the rest of the body, during the latter half of the cardiac cycle, and it is comprised of sounds resulting from the rise and release of pressure within the left ventricle along with the increase in ascending aortic pressure . After blood leaves the ventricles, the simultaneous closing of the semi lunar valves, which connect the ventricles with the aorta and pulmonary arteries, causes the second heart sound. The Electrocardiogram (ECG) represents the depolarization and repolarization of heart muscles during each cardiac cycle, (Gao, 2011). Depolarization of ventricular muscles during ventricular contraction results in three signals known as the Q, R, and S-waves of the ECG .The first heart sound immediately follows the QRS complex. In health, the last30–40% of the interval between successive R-wave peaks contains a period that is void off instant second heart sounds. Characteristics of heart sound signals have been assessed in terms of both intensity and frequency. Though peak frequencies of heart sounds have been shown to be much lower than those of lung sounds, comparisons between lung sound recordings acquired over the anterior right upper lobe containing and excluding heart sounds show that PSD in both cases is maximal below150 Hz.(Xie, 2019).

MATERIAL AND METHODS

A New Approach using the HS Filtering Technique: The method was analyzed using Advanced Signal Processing tool kit of LabVIEW. Lung sound is separated from Heart recordings using wavelet analysis tools and time series analysis tools. The Discrete Wavelet Transform (DWT) is applied to the recorded RS signal and locates the HS segments automatically and accurately using multiscale products. Initially, detection, localization and cancellation of HS included segments are performed using wavelet analysis tools. Time series analysis tools are used in modeling and predicting the HS removed portions of original RS Signal decomposition is done by wavelet functions. For each wavelet, wavelet transform calculates the inner products of the analyzed signal and localized in both time and frequency domains, so non-stationary signals are

processed only using wavelet signal processing. Based on adaptive time frequency resolution of wavelet signal processing, multi resolution analysis on non-stationary signals is carried out. Simultaneous long term and short term variations can be studied using wavelet transform-based multiscale analysis,(Palanivel Rajan, 2014) Multi scale product of wavelet approximation coefficients is computed in detecting the heart sounds. In wavelet decomposition three scales were used by considering fifth-order Symlet wavelet as the mother wavelet. The behavior of signal and noise is totally different in the wavelet domain. Concept of Lipschitz regularity is used in analyzing the behavior of signal and noise. The singularities are identified by multiplication of the DWT coefficients between the decomposition levels. In HS segment detection in RS signal is done by obtaining the multi scale product of the wavelet coefficients.

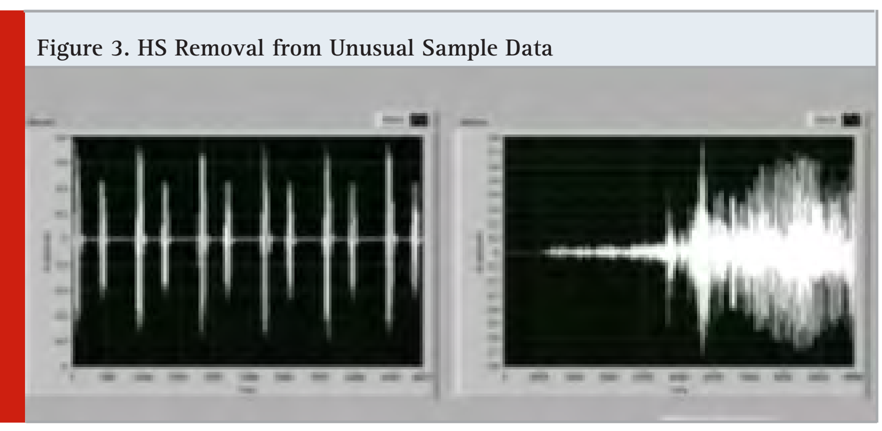
The specified level of the signal is decomposed by Multi resolution Analysis VI and signal reconstruction is performed for the selected frequency bands (Sathesh, 2012) The next step proposed is the HS localization. This Approach relies on the different behavior presented by the HS and RS across the wavelet scales. It uses the multi scale product at level 3 as a measure to detect singularities that in our case represent HS– included segments within the RS record. It is important to mention that the segment size that includes HS and is removed in this HS cancellation method is variable instead of being fixed sized blocks.(Thato, 2007). Wavelets functions are used in signal decomposition. The inner products of the analyzed signal and a family of wavelets are computed using wavelet transform. In time and frequency domain, wavelets are localized. So wavelet signal processing is best suited for non-stationary signals.

RESULTS AND DISCUSSION

During breathing lung sounds propagate through the lung tissue and can be heard over the chest wall. The tissue acts as a frequency filter with special characteristics based on Pathological changes. Therefore, auscultation and acoustical analysis of lung sounds are primary diagnostic assessments for respiratory diseases. The main components of Heart Sounds (HS) are in the range 20–100Hz, in which the lung sound has major components. High Pass Filtering (HPF) with an arbitrary cut-off frequency between70and100Hz is not efficient in this case because lung sounds have major components in that region particularly at low flow rates. Therefore, HS reduction from lung sounds without altering the main characteristic features of the lung sound has been of interest for many researchers (Tung, 2011).

There are dissimilar methods that have been applied for filtering heart sounds from lung-sound recordings as those that require a separate noise reference and those that do not, namely linear adaptive filters and filters employing time-frequency based methods respectively. These methods are compared on the basis of reduction in heart sounds and alteration to lung sounds by filtering. There are four main components to a linear adaptive filter: the input or "reference" signal; the output of the adaptive filter; the desired filter response or "primary" signal; and the

Figure 3. HS Removal from Unusual Sample Data

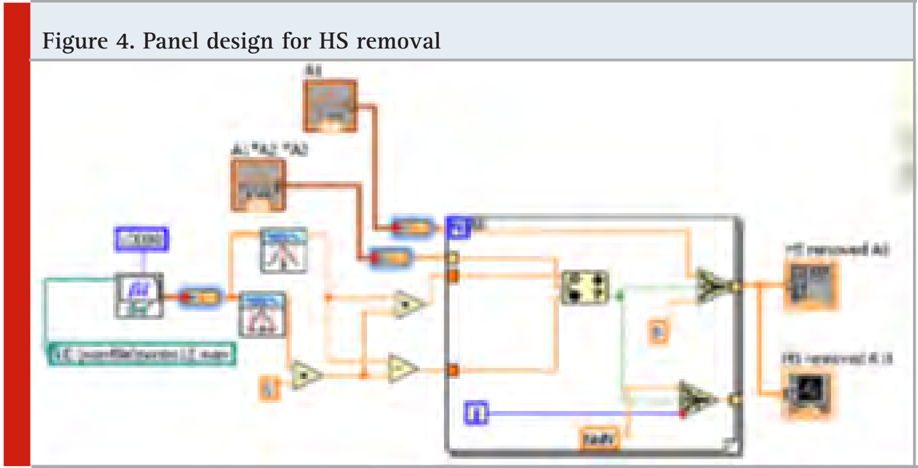


estimation error, which is the difference between the filter output and desired response. The term "linear" refers to the physical implementation of a linear adaptive filter, which employs the principle of super position between its input and output signals. In reality, the internal structure of a linear adaptive filter is highly non linear. A recursive algorithm within the adaptive filter updates filter parameters with each iteration (in discrete time operation) so as to minimize the estimation error. Noise cancellation and linear prediction are the two main classes of linear adaptive filters that have been applied to lung sound recordings for reducing heart sounds. Linear prediction serves to develop a model of a signal based on its past or future values. In adaptive noise cancellation, the primary input contains both the noise to be removed by the adaptive filter, and the signal of interest.

The reference signal represents the noise portion of the primary input; thus, the filter output is a signal that models the noise in the input, and the signal of interest is determined by subtracting the filter output from the primary input. Following schemes of adaptive filters are used: Least Mean Squares (LMS), Fourth-Order Statistics (FOS), Recursive Least Squares (RLS), Block Fast Transversal (BFT), and Reduced Order Kalman (ROK). Short-Time Fourier Transform (STFT) and wavelets provide means of examining signals simultaneously in both time and frequency domains. STFT or spectrogram, presents intensity of data within segments of constant time and frequency resolution. If data are non-stationary, window sizes must be chosen such that data within the windows are at least wide-sense stationary. This limits window size and hence spectral resolution. Wavelet Transform (WT) analysis provides more flexibility in terms of resolution and does not require that data be stationary. To localize heart sounds, the spectrogram was threshold within 10–110Hz per breath phase using a threshold incorporating the mean and standard deviation of average power calculated for both inspiratory and expiratory lung sounds (separately).Segments that corresponded to heart sounds were either completely removed from the spectrogram, or band-stop filtered using a 20–300 Hz band; in each case, gaps were created. Two dimensional interpolations, accounting for frequency and phase, were applied within each gap to recreate lung sounds based on the lung sounds occurring before and after the gap.

Time domain signals were reconstructed using inverse STFT, and results were assessed using PSD comparisons between filtered and unfiltered data. A study involving heart-sound localization and removal from lung sounds using WT also employed complete elimination of regions containing heart sounds as determined via thresholding. Three scales were used in wavelet decomposition with the fifth-order Symlet wavelet as the mother wavelet. The product of two adjacent decomposition bands, or the "multi scale product," was calculated for heart sound localization, and five standard deviations plus the mean value of lung sounds outside of heart-sound regions defined the threshold

Figure 4. Panel design for HS removal



for heart-sound localization. After removal of regions within each wavelet resolution level that corresponded to multi scale products that exceed the threshold, the lung sounds within these regions were modeled using either AR or MA modeling. Wavelet-like multi resolution analysis for heart-sound localization was applied in another study in which sub bands were created by filtering lung-sound recordings using half band low pass and high-pass filters and decimating each filtered sequence by a factor of two. This procedure was repeated for each low-pass-filtered sub band, creating N levels, each successive level having half the bandwidth, twice the frequency resolution, and half the time resolution of its predecessor, which is what results from WT as well. Using the time delay estimation scheme based on BFTF (block fast transversal filtering), time delays were determined within sub band levels four and five, which consistently matched. This delay was then applied to each of eight sub band of the reference heart-sound signal, and eight corresponding sub bands of the primary input (lung sounds containing heart sounds) were adaptively filtered for heart-sound removal. The filter output and error signals per sub band were reconstructed using an inverse scheme.

Daubechies quadrature mirror filters of eight coefficients were used for WT calculation in two studies employing wavelets for the removal of heart from lung sounds. In that method, (Huang, 2019) a threshold was applied to wavelet coefficients within each of 11 scales, which was defined by the standard deviation of the WT multiplied by a constant adjusting multiplicative factor chosen experimentally based on data recordings from individual subjects. The coefficients were separated per scale as those larger than the threshold and those smaller than the threshold. Reconstruction of coherent and residual signals from these groups of coefficients was performed across scales, representing the heart sounds (noise) and lung sounds (denoised data), respectively. The procedure was repeated in an iterative manner on the residual signals until the difference between the expected values of two re-successive residuals was less than a small constant (chosen as 0.00001). This stopping criterion implies that these waveforms were correlated and any coherent signal was no longer significantly present in (Huang, 2019). After the last iteration, the coherent parts obtained at each iteration of the procedure were summed, forming the heart sounds removed from lung sounds. As the first step of HS cancellation, the LS segments including HS must be identified. The premise of the proposed method in this paper is the HS localization technique. This approach relies on the different behaviors presented by the HS and LS across the wavelet scales. It uses the multi scale product at level3 to detect singularities that in our case represent HS-included segments within the LS record. It is important to mention that the segment size that includes HS and is removed in this HS cancellation method is variable instead of being fixed sized blocks. This algorithm is explained in detail in (Mayorga, 2019). Once the HS segments were localized and removed from the set off wavelet coefficients, the next step is to estimate the removed data. The length of the removed segment was different depending on the detected HS segment above the threshold. On average it was 100ms. In (Babu, 2019), estimating the removed data is done by linear prediction, using either Autoregressive (AR) or Moving Average (MA) models. A Rand MA models are two common signal processing tools used to predict past or future values of a time-limited signal.

The predicted samples are basically weighted linear combinations of the signal known values. It is too simple to as some that lung sounds are stationary during the entire duration of are aspiratory cycle (inspiration/expiration) especially at the vicinity of the onset so breathe. Therefore, correct selection of the order as well as the type of linear prediction model (AR or MA) must be done carefully to ensure the data used for prediction of the gaps are indeed stationary (Babu, 2019). In this paper, regardless of the flow rate, ARMA modeling and prediction is used. The input wave shape of original and abnormal recorded sample is shown in figure 2 is employed in investigation the heart sounds by getting the multiscale product of ripple approximation coefficients as shown in figure 3. Figure 4 shows the design of front panel for cancellation of heart sounds from respiratory sounds linear predictive filter. Localization of heart sound in original signal and abnormal

signal by from the singularities within the HS segments were performed by linear prediction and modeling. It is found that Adaptive filtering and use of wavelets are the best methods for locating heart sounds without noise.

CONCLUSION

Once HS segments are identified then the medical experts can easily identify the prone diseases. We are planning to develop a simple model using LabVIEW since LabVIEW is a graphical programming environment used by millions of engineers and scientists to develop sophisticated measurement, test, and control systems using intuitive graphical icons and wires that resemble a flowchart. The software tool implemented in the design for data acquisition is LabVIEW, selected primarily for its vast graphical capabilities and flexibility in design.

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A Fuzzy Model for Minining Amino Acid Assosiations In Peptide Sequences of Flavivirus Sub Families

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ABSTRACT

The Genus Flavivirus cause significant human disease in the form of encephalitis or hemorrhagic fever. This genus of the family Flavi viridae comprises of 70 viruses, but vaccines are available for only yellow fever, Japanese and Tick Borne Encephalitis. Disease diagnosis can be difficult as all the members of Flaviviridae are antigenically and genetically closely related. Thus it is important to reveal relationships between amino acids and other parameters in molecular sequences of Flavivirus as it may assist in controlling of the diseases caused by these viruses. In this paper an attempt has been made to develop and explore a model for mining fuzzy amino acid association patterns in peptide sequences of Flavivirus and their relationships with secondary structures and physicochemical properties. The uncertainty arising due to variation in length of sequences and this is handled by employing fuzzy sets. A tool based on fuzzy approach was developed to find fuzzy amino acid association patterns by calculating support and confidence. It also calculates secondary structure and physicochemical properties of amino acid association patterns. Total 9160 sequences were taken from National Centre for Biotechnology Information. After that around 4004 non-redundant peptide sequences of Flavivirus subfamilies filtered to form the dataset. This dataset is transformed to fuzzy transaction dataset and their fuzzy support and confidence have been computed. The association patterns generated from this model can be useful in understanding the structure, function and interaction of the protein in the disease. This patterns generated may also be useful in gaining better insight about the structure and function of the genus leading to development of new vaccines.

KEY WORDS: *CONFIDENCE, FUZZY ASSOCIATION MINING, DATASET, SUPPORT, THRESHOLDETHANOL.*

ARTICLE INFORMATION

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INTRODUCTION

Flaviviruses of the family Flavi viridae are important arthropod-borne viruses in both human and veterinary medicine. The Flavivirus family contains many viral agents which produces encephalitis. Flavivirus encephalitis's are either mosquito- borne, tick-borne, or an unknown vector (Oya and Kurane 2007). Major symptoms include mild acute febrile syndromes, severe neurological, hepatic and hemorrhagic disease. The geographical diversity of Flavivirus has shown the occurrence of Japanese Encephalitis Virus (JEV) in Asia, causing menigo encephalitis in children and West Nile Virus (WNV) in West Africa, Middle East, and from 1999 in North America (Blitvich 2008). The overview of the host genes and variants on modify susceptibility or resistance to major mosquito-borne flaviviruses infections in mice and humans (Manet and Roth 2018). Mosquito-borne flaviviruses and their interactions with the innate immune response have been well-studied and reviewed extensively, thus this review will discuss tick-borne flaviviruses and their interactions with the host innate immune response (Lindqvist and Upadhyay 2018).

Flaviviruses exploit the ER function during infection to gain optimal replication. Multiple independent genome-wide screen studies have identified several ER-associated complexes and individual proteins that are important for flavivirus replication. Thus, these ER- complexes represent promising host targets for developing broad-spectrum anti-flavivirus drugs,(Rothan and Kumar 2019). The area of bioinformatics is known for association analysis, which is one of the most popular analysis paradigms in data mining (Gupta et al. 2009). The association rule mining has become one of the core task, and motivated tremendous interest among the data mining researchers and practitioners (Agrawal et al. 1995). The association rule mining research mainly focuses on discovery of patterns and algorithms. The first reported algorithm for finding frequent item sets is the Apriori algorithm (Agrawal and Srikant 1994).

Since then a good number of algorithms are reported in the literature for association rule mining. The traditional association rule mining algorithms lack in capability of handling inherent uncertainties present in the biological data. Thus there is high possibility of generation of over predicted or under predicted patterns in the data. The fuzzy set approach can be employed for mining association pattern in molecular sequences to overcome this challenge to some extent (Zadeh 1965). Association rules read the nature of different amino acids that are present in the protein. This very basic analysis provides understandings into the Co-occurrence of certain amino acids in a protein (Gupta Mangal et al. 2006). Attempts are also reported in the literature for mining associations in molecular sequences. In this paper an attempt has been made to explore fuzzy amino association patterns in peptide sequences of Flavivirus. To develop a model for mining amino acid association patterns in peptide sequences of MTBC has been discussed. The variation in the length of these sequences leads to variation in degree of relationship among amino acids present in each sequence. The fuzzy set is employed to model this uncertainty of degree of relationships among the amino acids of the peptide sequences of MTBC (Seth and Pardasani 2014).

An approach for mining fuzzy association patterns in peptide sequences of dengue virus employed to incorporate the degree of relationships among amino acids due to variation in length of the sequences. This approach is employed to incorporate the relationship of parameters with amino acid association patterns (Gour and Pardasani 2018). Analytical Study of Data Mining Applications in Malaria Prediction and Diagnosis. This study shows the large number of deaths occur annually as a result of many factors which include shortages of medical personnel, laboratory equipment, hospitals and wrong interpretation of laboratory results. It also established the fact that remote areas are majorly affected. The fusion of Medical Science and Computer Science (Information Technology) in managing deadly diseases as a result of the earlier mentioned challenges was also established. This collaboration has led to development of computer based predictive models in medical diagnosis and treatment (Boruah and Kakoty 2019). Protecting the Privacy of Cancer Patients Using Fuzzy Association Rule Hiding, a novel method was presented to hide the sensitive rule in quantitative data by decreasing the support of the RHS of the rule. Experimental results demonstrate that the proposed approach is more efficient as it facilitates better rule hiding and minimizes the number of lost rules and ghost rules. Also, this approach makes minimum modifications to the

dataset (Krishnamoorthy and Murugesan 2018, Hussain and Kumar 2019).

In this study a Java EE platform based tool was developed of for studying of molecular sequences. The main feature of tool is its accuracy and intelligence in generating the results. The main aim is to analyze the fuzzy associations between various frequent patterns occur to handle upcoming challenges of uncertainty. The available bioinformatics tools provide information only about the secondary structure and physicochemical properties of entire peptide sequences without using any parameter like length of the sequences, length range of the sequences, creating difficulty in critical analysis due to under prediction and over prediction of the rules. The divergence and convergence of association patterns within the Flavivirus subfamilies is analyzed to generate the association rules. The results generated are also correlated with structural and physicochemical properties.

MATERIAL AND METHODS

Description of the algorithm employed is as follows: In this paper we have taken molecular data of Flavivirus subfamilies like: mosquito borne, Tick borne, Known vector from NCBI. To calculate the Fuzzy frequent patterns in redundant and non-redundant dataset of Flavivirus subfamilies, the fuzzy membership of amino acid in respective sequence is calculated as

$$\mu_i(A)=\sum_i^n f_i(A)/L_i \quad (1)$$

Where $\sum_i^n f$ (A) is the frequency of amino acid A and μ_i (A) is the membership of amino acid A in the ith sequence. It is assumed that there are 20 amino acids and each amino acid will have equal likely chance of appearing in a sequence. Thus the threshold value can be calculated as:

$$T=0.05*N \quad (2)$$

Here N is the Number of sequences.
The apriori algorithm is employed to find frequent patterns in all the sequences. These patterns are used to generate association rule .The Fuzzy Support from amino acid can be calculated as: The frequency Support for n amino acid can be calculated as:

$$\sum \mu_i(A1 \cap A2 \cap A3 \cap \ldots\ldots\ldots\ldots\ldots A_{n-1} \cap A_n) \quad (3)$$

Confidence for n amino acid can be calculated by:

$$\frac{\sum_i^L \mu i(A1 \cap A2 \cap A3 \cap \ldots\ldots\ldots\ldots An-1 \cap An)}{\sum_i^L \mu i(A1 \cap A2 \cap A3 \cap \ldots\ldots\ldots\ldots An-1)} \quad (4)$$

RESULTS AND DISCUSSION

After applying the fuzzy approach for finding

Step 1:		
Sequence number (Transactions)	**Amino Acids(Item Set)** a_1 a_2 .... a_{19} a_{20}	**Length of Sequence (Parameter)**
s_1 s_2 . s_n	$a_{1.1}$ $a_{1.2}$ .... $a_{1.19}$ $a_{1.20}$ $a_{2.1}$ $a_{2.2}$ .... $a_{2.19}$ $a_{2.20}$. $a_{n.1}$ $a_{n.2}$ .... $a_{n.19}$ $a_{n.20}$	l_1 l_2 . l_n
Step 2:		
Sequence number (Transactions)	**Membership of Amino Acids(Item Set)** a_1 a_2 .... a_{19} a_{20}	**Sequence number (Transactions)**
s_1 s_2 . s_n	$\mu_1(a_1)$ $\mu_1(a_2)$ .... $\mu_1(a_{19})$ $\mu_1(a_{20})$ $\mu_2(a_1)$ $\mu_2(a_2)$ .... $\mu_2(a_{19})$ $\mu_2(a_{20})$. $\mu_n(a_1)$ $\mu_n(a_2)$ .... $\mu_n(a_{19})$ $\mu_n(a_{20})$	s_1 s_2 . s_n
Step 3:		
Sequence number	**Amino Acids(Item Set)** a_1 a_2 .... a_{19} a_{20}	**Length of Sequence (Parameter)**
s_1 s_2 . s_n	$a_{1.1}$ $a_{1.2}$ .... $a_{1.19}$ $a_{1.20}$ $a_{2.1}$ $a_{2.2}$ .... $a_{2.19}$ $a_{2.20}$. $a_{n.1}$ $a_{n.2}$ .... $a_{n.19}$ $a_{n.20}$	l_1 l_2 . l_n

the maximum and minimum frequency of each amino acid for all subfamilies of Flavivirus, it has been found that there are variations in frequent amino acid for redundant and non-redundant data set of all three subfamilies of Flavivirus :-mosquito borne, tick borne and known vector subfamilies. G(glycine) is most frequent amino acid for Japanese encephalitis, St. Louis encephalitis , West Nile, Louping ill and Summar encephalities while L((luecine) is most frequent for Marry Valley, Ilheus, ,Central European, Russion Spring-Rodant, Ricio and Rio Bravo whereas V(valine) amino acid is most frequent for Powassion. Amino acid C (cystein) is least frequent in Marry Vally, Ilheus , West Nile, Louping Ill, Russin Spring-Rodant, Summar encephalitis, Ricio and Rio Bravo whereas amino acid H(Histidine) is least frequent in Japanese encephalitis, St. Louis encephalitis, and Powassion ; and W(Tryptopher) is least frequent for Central European. Table 1 shows the maximum support for frequent amino acid among the subfamilies of mosquito borne family of Flavivirus. A, G, L, T and S (Alanine, Glycine, Leucine, Threonine

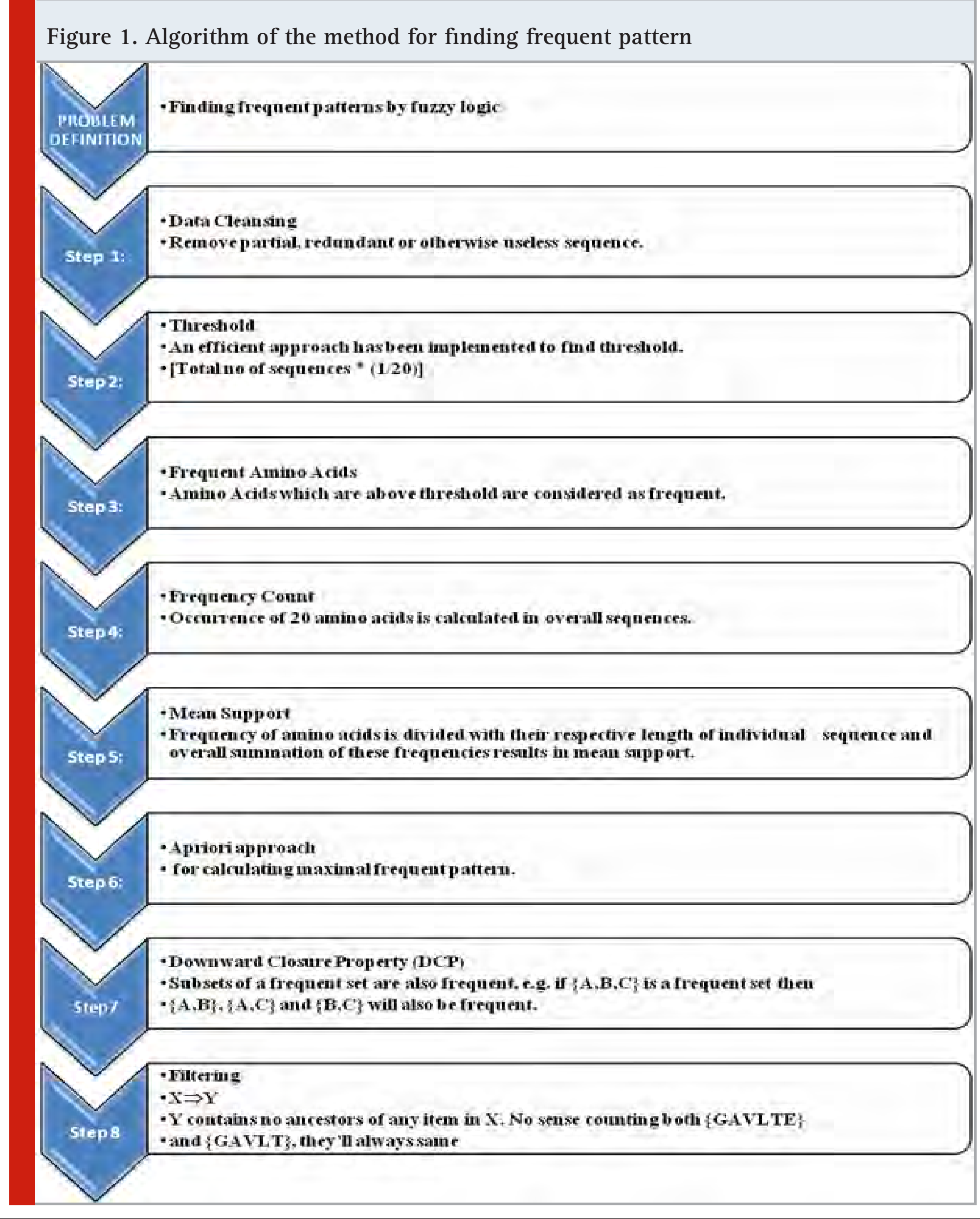


Figure 1. Algorithm of the method for finding frequent pattern

Table 1. Maximum support in case of Mosquito Borne

Japanese encephalitis		Marry Vally encephalitis		St. Louis encephalitis		Ilheus		West Nile	
R	Non-R	R	Non-R	R	Non-R	R	Non-R	R	Non-R
S OF SIX FP [G, A, L, T, V, S]	S OF SIX FP [G, A, L, T, V, S]	S OF EIGHT FP[E, G, A, L, K, T, V, S]	S OF SEVEN FP[E, G, A, L, K, T, V, S]	S OF SEVEN FP[G, A, L, K, T, V, S]	S OF SEVEN FP[E, G, A, L, K, T, V, S]	S OF FIVE FP[F, G, A, L, I, T, S]	S OF FIVE FP[G, A, L, I, T, V, S	S OF SIX FP[G, A, L, T, V, S]	S OF FIVE FP[G, A, L, T, V, S]
GALTVS	GALT	EGALKT	GALK TVS	GALKTVS	GALKTVS	GALIS	GALIS	GALTVS	GALTV
S= 127.35	VS S = 55.66	VS S= 5.29	S = 3.01	S = 13.93	S = 9.30	S= 8.22	S=4.28	S= 203.74	S= 115.42
TOTAL NO OF SIX	TOTAL NO OF SIX	TOTAL NO OF EIGHT	TOTAL NO OF SEVEN	TOTAL NO OF SEVEN	TOTAL NO OF SEVEN	GALTS S = 8.16	TOTAL NO OF FIVE	TOTAL NO OF SIX	TOTAL NO OF FIVE
FP: 1 -	FP: 1 -	F P: 1 -	FP: 1 -	FP: 1 -	FP: 1 -	GLITS	FP : 1	FP : 1	FP: 1
-	-	-				S= 8.25			
- -	- -	- -	-	-	-	ALITS S= 8.09			
-	-	-	-	-	-	TOTAL NO OF FIVE FP: 4	-	-	-

*S= Support, FP= Frequent Patterns

Table 2. Maximum support in case of Tick Borne

Central European (4033)		Louping Ill (45)		Powassan (126)		Russian Spring-Rodents (7)		Summer encephalitis 2)	
R	Non-R	R	Non-R	R	Non-R	R	Non-R	R	Non-R
S OF FOUR FP [G, A, L, I, T, V, S]	S OF FOUR FP [G, A, L, I, T, V, S]	S OF FIVE FP [E, G, A, L,K, T, V, S, R]	S OF FIVE FP [E, G, A, L, K, T, V, S, R]	S OF SIX FP [D, E, G, A, L, K, T, V, S]	S OF SIX FP [E, G, A, L, K, T, V, S, R]	S OF SIX FP [G, A, L, I, T, V]	S OF FIVE FP [E, A, L, I, K, T, V, R]	S OF EIGHT FP [D, E, G, A, L, V, S, R]	S OF EIGHT FP [D, E, G, A, L, V, S, R]
GALI S = 203.74	GALV S = 127.43	EGALS S = 2.30	EGALS S = 1.76	EGATVS S = 6.32	EGALVS S= 3.81	GALITV S = 0.36	EALKR S = 0.29	DEGAL VSR S = 0.10	DEGAL VSR S =0.10
GALV S = 218.84	GALS S=123.58	GALTV S= 2.86	GALTV S= 2.22	EAKTVS S= 6.53	TOTAL NO OF SIX FP: 1	TOTAL NO OF SIX FP: 1	ALITV S = 0.26	TOTAL NO OF EIGHT FP: 1	TOTAL NO OFEIGHT FP: 1
GALS SUPP ORT= 209.47	ALVS SUPPORT = 121.30	TOTAL NO: OF FIVE FP:2	TOTAL NO OF FIVE FP: 2	GAKTVS S = 6.40	-	-	TOTAL NO OF FIVE FP: 2	-	-
TOTAL NO OF FOUR FP: 3	TOTAL NO OF FOUR FP:3	-	-	ALKTVS S = 6.30	-	-	-	-	-
-	-			TOTAL NO OF SIX FP: 4	-	-	-	-	-

and Serine) are frequent for all subfamilies. V (Valine) is also frequent for all subfamilies except of Ilheus virus for non-redundant dataset. E (Glutamic) is frequent in Marry Valley for both redundant and non-redundant dataset and in St. Louis encephalitis (non-redundant dataset). K (Lysine) is frequent in marry valley and St. Louis encephalities. I (Isoleucine) is frequent for Ilheus. F (Phenylalanine) is frequent for redundant dataset of Ilheus virus. Table 2 represents maximus support found for tick borne subfamily. In tick borne subfamily maximum support for frequent amino acid are A, L, V (Alanine, Leucine,

Valine) for all subfamilies. G(Glycine) is frequent for all subfamilies except the Russion Spring-Rodant non-redundant dataset. T (Threonine) is also frequent for all subfamilies except Summer encephalitis. S (Serine) is frequent for all subfamilies except Russion Spring-Rodent dataset. E (Glutamic) is frequent for Louping Ill, Powassan , Summer encephalitis and Russion Spring-Rodent (non-redundant datase). K(Lysine) is frequent for Louping Ill, Powasson and Pussion Spring-Rodent (non-redundant dataset),R is frequent for Louping Ill, Powassan (non-redundant dataset) and Russion Spring-Rodent(non-redundant dataset), I is frequent for Central european and Russion Spring-Rodent, D (Aspartic) is frequent for Summar encephalitis and Powassan (redundant dataset).

Table 3 depicts that in known vector subfamilies of Flavivirus; L,I,T,V,S (Leucine, Isoleucine, Threonine, Valine, Serine) are frequent for all subfamilies, G(Glycine) and A(Alanine) are frequent for Rico and Rio-bravo (redundant dataset), S(Serine) is also frequent for Racio and K(Lysine) is frequent for Rocio non-redundant dataset. Kumari and Pardasani (2013,14), have applied the same method in their research but with different dataset i.e., GPCRs. Table 4 shows the probable structure (helix, beta, and coil) and physicochemical properties of Favivirus subfamilies: Mosquito Borne, Tick Borne and Known Vector. The observation reveals that the most of the amino acids G, A, L (Glycine, Alanine, Leucine) are common in all subfamilies and Helix is the Probable Structure in maximum subfamilies. The physicochemical properties like hydrophobicity, CBetaBranched, polar aliphatic and uncharged, non-polar aliphatic groups are common in all subfamilies. Table 5 shows the probable helix structure of protein for Flavivirus subfamilies based on amino acid associations. Amino acids like A, R, E, Q, L, K, M, H are responsible for helix structure formation. It has been revealed that the maximum frequent patterns for helix formation are 4-frequent patterns. Table 6 shows the probable sheet structure of protein for Flavivirus subfamilies based on amino acid associations. Amino acids like V, I, T, C, W, F, Y are responsible for secondary sheet structure formation.

Table 3. Maximum support in case of Known Vector

Rocio R	Non-R	Rio Bravo R	Non-R
S OF THREE FP [G, A, P, S] I, T, V, P, S]	S OF THREE FP [G, A, L, I, K, T, V, P, S]	SOF FOUR FP [G,A, L, I,T, V,S]	S OF FOUR FP [L, I, T, V, S]
GLI S = 18.40	GLI S= 16.64	LIVS S = 2.43	LIVS S = 1.75
GLT S = 17.54	GLT S = 15.79	TOTAL NO OF FOUR FP: 1	TOTAL NO OF FOUR FP: 1
GLV S = 18.43	GLV S = 16.62	-	-
GLP S = 17.86	GLP S = 16.20	-	-
GLS S = 17.45	GLS S = 15.68	-	-
TOTAL NO OF THREE FP:5	TOTAL NO OF THREE FP:5	-	-

It has been observed that the maximum frequent patterns are 3 for secondary structure of sheet formation i.e. ITV. Table 7 presents the secondary structure of coil formation in which almost all subfamilies have 2-frequent patterns GS which are responsible for formation of secondary structure. It has been observed in Table 5, 6 and 7 that in all the 12 subfamilies of Flavivirus association patterns of amino acid exposed high tendency to form secondary structure Helix rather than Sheet and Coil. Table 8 and Figure 1 and 2 depict the percentage wise calculations of physicochemical Properties of mosquito borne subfamilies. It reveals that west Nile virus (non-redundant dataset) have high percentage of Molecular weight and Extension Coefficient among all the subfamilies of mosquito borne.

Redundant dataset of West Nile virus have shown higher tendency to form a secondary structure Coil (29.135%) among all the subfamilies of Mosquito Borne. Marry Vally has shown high tendency of Absorbance among all the subfamilies. All the subfamilies of Mosquito borne viruses show negative hydrophobicity. Illeus virus has high percentage of aliphatic index and Aromaticity among all the subfamilies of mosquito borne virus. C-Beta Sheets are higher in Japanese encephalitis redundant dataset. Protein stability is high for Marry Vally virus in redundant and non-redundant dataset among all the subfamilies of mosquito borne. In Louis encephalitis both (redundant and non-redundant) datasets are showing high Salt Bridged, Positive Charged, and

Table 4. Probable Structures and physicochemical Properties of Protein Sequences of Sub families

Subfamily	R F- Amino Acid	Non-R Probable Structure	Physicochemical Properties	F- Amino Acid	Probable Structure	Physicochemical Properties
Mosquito Borne Subfamilies						
Japanese encephalitis	G, A, L, T, V, S	Helix, Sheet and Coil	Polar aliphatic (G), polar uncharged(S, T), non-polar aliphatic(A,L,V) and hydrophobic (G,L,V), CBeta Branched(T,V)	G, A, L, T, V, S	Helix	Acidic Negative charged protein stable (E), Polar aliphatic (G),polar uncharged(S,T), non-polar aliphatic(A,L,V) and hydrophobic (G,L,V), CBeta Branched(T,V)
Marry Vally encephalitis	E, G, A, L, K, T, V, S	, Helix	Acidic Negative charged protein stable(E), Polar aliphatic (G), non-polar aliphatic(A,L,V), and polar uncharged(T,S), basic charged (K) and hydrophobic (G,L,V), CBeta Branched(T,V)	E, G, A, L, K, T, V, S	Helix	Acidic Negative charged protein stable (E), Polar aliphatic hydrophobic (G), non-polar aliphatic(A,L,V), polar uncharged (T,S),basic charged(K) and hydrophobic(G,L,V), CBetaBranched (T,V)
St. Louis encephalitis	F,G, A, L, I, T, V,S	Helix & Sheet	Aromatic, Aliphatic(F) Polar aliphatic (G),polar uncharged (T,S),non-polar aliphatic (A,L,V,I) and hydrophobic (G,L,V,F), CBetaBranched(T,V)	G, A, L, I, T, V,S	Helix	Polar aliphatic (G), polar uncharged(T,S), non-polar aliphatic(A,L,V,I) and hydrophobic(G,L,V), CBetaBranched(T,V)
Ilheus	G, A, L, T, V, S	Sheet	Polar aliphatic (G), polar uncharged(S, T), non-polar aliphatic(A,L,I,V) and hydrophobic(G,L,V) CBetaBranched(T,V)	G, A, L, T, V, S	Sheet	Polar aliphatic (G), polar uncharged(S, T), non-polar aliphatic(A,L,V) and hydrophobic(G,L,V), CBetaBranched(T,V)
West Nile	G, A, L, T, V, S	Helix	Polar aliphatic (G), non-polar aliphatic(A,L,V), and polar uncharged(T,S) and hydrophobic(G,L,V), CBetaBranched(T,V),	G, A, L, T, V, S,	Helix	Polar aliphatic (G), non-polar aliphatic (A,L,V), and polar uncharged(T,S) and hydrophobic(G,L,V), CBetaBranched (T,V),
Tick Borne Subfamilies						
Central European	G, A, L, T, V, S	Helix, Sheet and Coil	Polar aliphatic (G), polar uncharged(S, T), non-polar aliphatic(A,L,V) and hydrophobic(G,L,V), CbetaBranched(T,V)	G, A, L, T, V, S	Helix	Acidic Negative charged protein stable (E), Polar aliphatic(G), polar uncharged (S,T),non-polar aliphatic(A,L,V) and hydrophobic(G,L,V), CbetaBranched(T,V)
Louping Ill	E, G, A, L, K, T, V, S, R	Helix	Acidic Negative charged protein stable(E), Polar aliphatic (G), non-polar aliphatic(A,L,V), and polar uncharged(T,S), basic charged(K) and hydrophobic(G,L,V), CbetaBranched(T,V), basic positive charged protein stable ®	E, G, A, L, K, T, V, S, R	Helix	Acidic Negative charged protein stable (E), Polar aliphatic hydrophobic (G), non-polar aliphatic (A,L,V), polar uncharged (T,S),basic charged (K) and hydrophobic(G,L,V), CbetaBranched (T,V), basic positive charged protein stable ®
Powassan	D,F,G, A, L, K, T, V,S	Helix & Sheet	Acidic Negative charged protein stable(E), Polar aliphatic (G),polar uncharged(T,S), non-polar aliphatic(A,L,V) and hydrophobic(G,L,V), CbetaBranched(T,V), basic charged(K), Acidic Charged (D)	E , G, A, L, K, T, V,S , R	Helix	Acidic Negative charged protein stable(E),Polar aliphatic (G),polar uncharged(T,S),non-polar aliphatic(A,L,V) and hydrophobic(G,L,V), CbetaBranched(T,V),

Russian Spring-Rodents	G, A, L, I, T, V	Sheet	Polar aliphatic (G), polar uncharged(T), non-polar aliphatic (A,L,I,V) and hydrophobic (G,L,V) CbetaBranched(T,V	E, A, L, I, K, T, V, R	Sheet	Basic charged amino acid®, basic charged(K), Acidic Negative charged protein stable(E),polar uncharged(S,T), non-polar aliphatic(A,L,I,V) and basic charged(K), hydrophobic (G,L,V), CbetaBranched (T,V), Basic charged amino acid®
Summer encephalitis	D,E, G, A, L, T, V, S,R	Helix	Acidic Charged (D), Acidic Negative charged (E) ,Polar aliphatic (G), non-polar aliphatic (A,L,V), and polar uncharged(T,S) and hydrophobic(G,L,V), CbetaBranched(T,V), Basic charged amino acid®, protein stable(D,R,E)	D,E, G, A, L, T, V, S,R	Helix	Acidic Charged (D), Acidic Negative charged protein stable (E),Polar aliphatic (G), non-polar aliphatic (A,L,V), and polar uncharged(T,S) and hydrophobic(G,L,V), CbetaBranched(T,V), Basic charged amino acid®, protein stable(D,R,E)
Known Vector subfamilies						
Rocio	G, A, L, I, T, V, P, S	Coil and Helix	Polar aliphatic (G), polar uncharged(T,S), non-polar aliphatic (A,L,I,P,V) and hydrophobic (G,L,V), CBeta Branched(T,V)	G, A, L, I, K, T, V, P, S (A,I,L,V,P)	Helix and Coil	Polar aliphatic (G), polar uncharged(S,T), non-polar aliphatic and, hydrophobic (G,L,V), CBetaBranched (T,V),basic charged (K)
Rio Bravo	G, A, L I,T, V,S	Helix, Coil	Polar aliphatic (G), polar uncharged(S,T), non-polar aliphatic (A,L,I,V), and hydrophobic (G,L,V), CBetaBranched(T,V)	L, I, T, V,S	Coil	non-polar aliphatic(L,I,V), polar uncharged(T,S) and hydrophobic(L,V), CBetaBranched(T,V)

Negative Charged parameters. Polarity is high in Marry Vally among all the subfamilies of mosquito borne of Flavivirus. Marry Vally shows the high tendency of Helix formation with respect to other subfamilies of Mosquito borne. Beta Sheet formation tendency is high in Japanese encephalitis redundant dataset than other subfamilies. Table 9 and Figure 3 and 4 present the physicochemical properties of tick borne subfamilies ,it has been found that non redundant dataset of Russian Spring-Rodent have high percentage of molecular weight ,extension coefficient ,positive charge, negative charge, salt bridged, polarity, protein stability and helix formation among all the subfamilies of Tick borne subfamilies, Central European redundant dataset are having high percentage of hydrophaticity, aromalicity and beta sheet formation. Among all subfamilies of tick borne, coil formation is high in non redundant dataset of Central European. Absorbance is high in Louping ill redundant dataset, C-Beta branched is high in Powassion, aliphatic index is high in Russian Spring-Rodent among all subfamilies of Tick Borne virus. Table 10 and Figure 5 & 6 present some physicochemical properties of known vector subfamilies. It has been found that molecular weight, aliphatic index, aromaticity, extension coefficient absorbance , C-Beta branched and helix formation are high in Rio bravo(redundant dataset) and beta sheet formation tendancy is high in Rio bravo non-redundant dataset. Hydrophaticily, polarity, salt bridged and coil formation are high in Rocio (redundant dataset) and protein stability and positive-negative charge are high in non-redundant dataset of Rocio. Some of the researcher have applied the same method in their research but with different dataset like Shankar and Pardasani (2013) worked on the dataset Apphaproteo bacteria; Seth Pardasani (2015), worked on the dataset MTBC; Gour and Pardasani (2018) studied the dataset Dengue Virus. The mathematical expressions represent the degree of relationships among amino acids in peptide sequences of flavivirus subfamilies and association relationships among amino acids in peptide sequences of flavivirus subfamilies(mosqitu born, tike born and known vector). These relationships are characterized by fuzzy membership, fuzzy support and fuzzy confidence. The relationships are interpreted in terms of associations rules of amino acids in peptide sequences.

The association rules generated on the basis of above Flavivirus subfamilies results are given below:-

Mosquito Borne Subfamilies:-
For 2 frequent Patterns:-

1. {A(Frequent)∩L(Frequent)=>Tendency for Helix Formation}
2. {A(Frequent)∩E(Frequent)=>Tendency for Helix

Table 5. Probable Helix Structure of Protein of Flavivirus Subfamilies based on amino acid association

Subfamily	Helix Formation (A,R,E,Q,L,K,M,H)							
	R				Non-R			
	1-FP	2- FP	3- FP	4- FP	1- FP	2- FP	3- FP	4- FP
Japanese encephalitis	A,E,L	AL	None	None	A,E,L,K	AL	None	None
Marry Vally encephalitis	A,L, E,K,R	AL,A E,AK, LE,LK, EK,ER	EAL, EAK, ELK, ALK ER	EALK	A,L, E,K,R	AL,AE, AK,LE, LK,EK,	EAL, EAK, ELK, ALK,	EALK
St. Louis encephalitis	A,L, E,K	AL,AE, AK,LE, LK,EK	ALK	None	A,L, E,K	AL,AE, AK,LE, LK,EK	EAL, EAK, ELK, ALK	EALK
Ilheus	A,L	AL	None	None	A,L	AL	None	None
West Nile	A,L, K	AL, LK	None	None	A,L, E,K	AL, LK	-	-
Central European	A,L, E,K	AL	None	None	A,L, E,K	AL	None	None
Louping Ill	A,L, E,K,R	AL,AE ,AK LE,LK, EK,AR. LR	EAL, ALK, ALR	None	A,L, E, K,R	AL, AE, AK LE,LK, EK, AR.LR	EAL, ALK, ALR	None
Powassan	A,L, E,K,R	AL, AE, AK, AR, LE,LK, LR , EK,ER	EAL, EAK, ALK, ALR	None	A,L, E,K,R	AL,AE, AK, LE,LK, EK, AR,LR	EAL, EAK, ELK, ALK	None
Russian Spring-Rodents	A,L, E,K	AL,AE, EK,LE	EAL	None	A,L, E,K,R	AL,AE, AR,AK, LE,LK, LR,EK, ER,KR	EAL, EAK, ELR, EKR, ALK, ALR, AKR, LKR	EALK, EALR, EAKR, ALKR
Summer encephalitis	A,L, R,E,K	AR,AK, AE,,LE, LK,LR, ER,AL	EAL, EAR, ELR, ALK, ALR	EALR	A,L, R,E,K	AR, AK, AE,,LE, LK,LR, ER,AL	EAL, EAR, ELR, ALK, ALR	-
Rocio	A,L,R,K	AL	None	None	A,L, K	AL,LK	None	None
Rio Brivo	A,L,K	AL,LK	None	None	A,L,K	AL,LK	None	None

Formation}

3. {E(Frequent)∩K(Frequent)=>Tendency for Helix Formation and Protein Solubility}
4. {E(Frequent)∩R(Frequent)=>Tendency for Helix Formation and Protein Solubility }
5. {V(Frequent)∩T(Frequent)=>Tendency for Sheet Formation}
6. G(Frequent)∩S(Frequent)=>Tendency for Coil Formation}

For 3 frequent Patterns:-

1. { E(Frequent)∩A(Frequent)∩L(Frequent)=>Tendency for Helix Formation}
2. {A(Frequent)∩L(Frequent)∩K(Frequent)=> Tendency for Helix Formation}

Table 6. Probable Sheet Structure of Protein of Flavivirus Subfamilies based on amino acid association

Subfamily	Sheet Formation (V,I,T,C,W,F,Y)							
	R				Non-R			
	1- FP	2- FP	3- FP	4- FP	1- FP	2- FP	3- FP	4- FP
Japanese encephalitis	V,T,I	VT,VI	None	None	V,T,I	VT,VI	None	None
Marry Vally encephalitis	V,T	VT	None	None	V,T	VT	None	None
St. Louis encephalitis	V,T,I	VT	None	None	V,T	VT	None	None
Ilheus	V,F,I,T	VI,VT,IF,IT	None	None	V,I,T	VI,IT	None	None
West Nile	V,T	VT	None	None	V,T	VT	None	None
Central European	V,T,I	VT,VI	None	None	V,T,I	VT	None	None
Louping Ill	V,T	VT	None	None	V,T	VT	None	None
Powassan	V,T	VT	None	None	V,T	VT	None	None
Russian Spring-Rodents	V,I,T	VI,VT,IT	ITV	None	V,I,T	VI,VT,IT	ITV	None
Summer encephalitis	V,T	VT	None	None	V,T	VT	None	None
Rocio	V,T,I	VT,VI	None	None	V,T,I	VT	None	None
Rio Brivo	V,T,I	VI,IT	None	None	V,T,I	VI,IT	None	None

Table 7. Probable Coil Structure of Protein of Mosquito Borne Subfamilies based on amino acid association

Subfamily	Coil (N,D,P,S,G)							
	R	Non-R						
	1- FP	2- FP	3- FP	4- FP	1- FP	2- FP	3- FP	4- FP
Japanese encephalitis	G,S,D	GS	none	none	G,S	GS	None	None
Marry Vally encephalitis	G,S	GS	None	none	G,S	GS	None	None
St. Louis encephalitis	G,S	GS	None	None	G,S	GS	None	None
Ilheus	G,S	GS	None	None	G,S	GS	None	None
West Nile	G,S	GS	None	None	G,S	GS	None	None
Central European	G,S	GS	none	none	G,S,D	GS	None	None
Louping Ill	G,S	GS	None	none	G,S	GS	None	None
Powassan	G,S,D	GS	None	None	G,S,D	GS	None	None
Russian Spring Rodents	G,S	None	None	None	G,S	G	None	None
Summer encephalitis	G,S,D	GS,GD, SD	DGS	None	G,S,D	GS,GD ,SD	DGS	None
Rocio	G,S,P	GS,GP	none	none	G,S,P	GS,GP	None	None
Rio Brivo	G,S	GS	None	none	G,S	None	None	None

Table 8. Physicochemical Properties of Mosquito Borne Subfamilies

Physicochemical Properties/ Parameters	Japanese encephalitis	Marry Vally encephalitis		St. Louis encephalitis		Ilheus	West Nile			
	R	Non-R	R	Non-R	R	Non-R	R	Non-R	R	Non-R
MOLECULAR WEIGHT	55938.88	97201.14	50446.12	61154.543	83670.14	99688.87	32957.98	33770.816	89999.94	111316.75
EXTENSION COEFFICIENT	87659.75	157137.25	81971.6	100050.16	131802.67	158459.83	53105.535	48602.41	144807.45	183429.42
ABSORBANCE	1.52	1.50	1.56	1.57	1.54	1.52	1.54	1.35	1.55	1.56
HYDROPHATICITY [GRAVY]	-0.04	-0.10	-0.30	-0.22	-0.18	-0.20	0.23	0.11	-0.13	-0.17
ALIPHATIC INDEX	86.74%	83.97%	80.47%	84.11%	78.93%	78.49%	102.01%	99.14%	82.76%	83.21%
AROMATICITY	8.22%	8.21%	8.65%	8.54%	8.85%	8.82%	10.86%	9.66%	9.17%	9.18%
PROTEIN STABILITY	22.97%	22.92%	24.90%	24.26%	22.55%	23.24%	16.67%	19.57%	21.78%	22.72%
C-BETA BRANCHED	2190%	21.18%	20.06%	20.67%	21.70%	21.27%	20.75%	19.95%	19.82%	19.61%
POLARITY	49.279%	49.60%	51.33%	50.29%	49.55%	49.96%	43.96%	45.30%	48.87%	49.28%
SALT BRIDGED	19.20%	19.68%	23.66%	22.58%	20.28%	20.61%	15.53%	17.68%	19.12%	20.01%
HELIX FORMATION	38.10%	39.04%	44.12%	43.46%	38.80%	39.43%	40.54%	41.52%	39.92%	40.72%
BETA SHEET	33.08%	32.02%	30.45%	30.89%	32.68%	32.39%	32.48%	30.77%	30.95%	30.68%
COIL	28.83%	28.94%	25.43%	25.65%	28.53%	28.19%	26.98%	27.71%	29.13%	28.60%
POSITIVE CHARGED	9.48%	10.10%	12.23%	11.84%	10.49%	10.72%	7.33%	8.88%	10.47%	11.00%
NEGATIVE CHARGED	10.52%	10.19%	10.93%	10.74%	9.93%	10.21%	8.46%	9.53%	9.35%	9.83%

Figure 1. Secondary structure Formation of Mosquito Borne Subfamilies

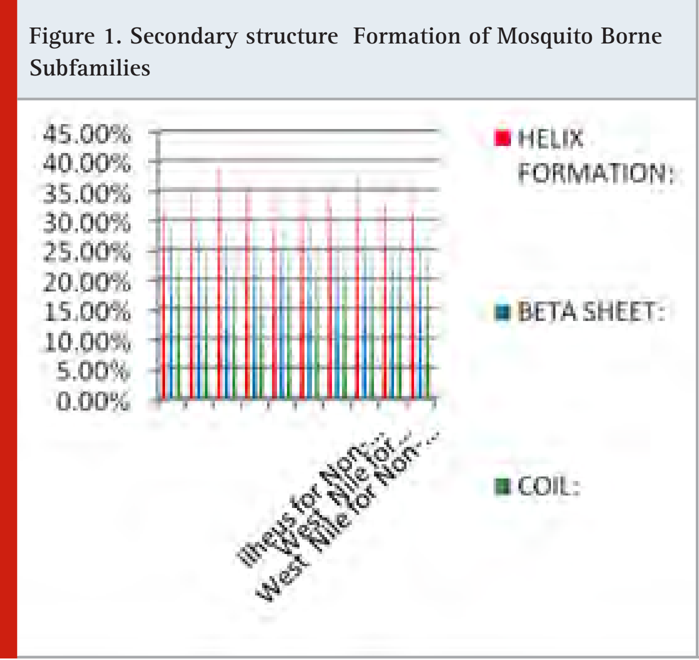


Figure 2. Protein stability of Mosquito of Borne Subfamilies

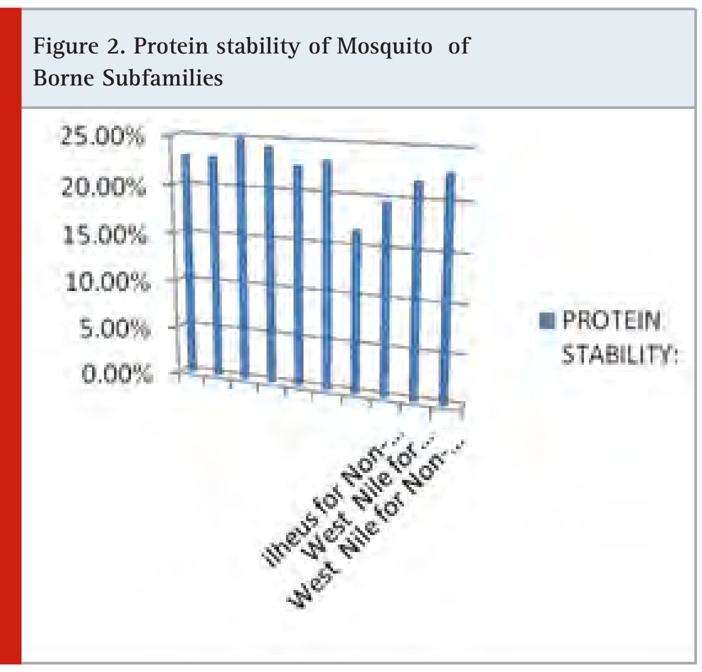


Table 9. Physicochemical Properties of Tick Borne Subfamilies

Physicochemical Properties (Parameters)	Central European		Louping l Il		Powassan		Russion Spring-Rodent	Summar encephalitis		
	R	Non-R	R	Non-R	R	Non-R	R	Non-R	R	Non-R
MOLECULAR WEIGHT	51949.90	64864.06	62048.28	48462.6	76665.56	104627.31	975770.7	1356700.0	46053.8	46053.8
EXTENSION COEFFICIENT	59115.016	73226.59	103324.78	79950.14	134810.8	188640.06	1096284.2	1520818.0	75842.5	75842.5
ABSORBANCE	1.20	1.17	1.75	1.74	1.62	1.75	1.02	0.83	1.71	1.71
HYDROPHATICITY GRAVY]	-0.04	-0.17	-0.09	-0.07	-0.31	-0.26	-0.04	-0.42	-0.21	-0.21
ALIPHATIC INDEX	91.54%	86.54%	87.55%	88.60%	77.86%	82.12%	98.51%	.15%	82.46%	82.46%
AROMATICITy	9.33%	9.00%	8.61%	8.41%	7.80%	7.95%	7.22%	5.72%	8.31%	8.31%
PROTEIN STABILITY	21.45%	23.28%	23.30%	23.36%	27.17%	26.40%	24.13%	.41%	24.73%	24.73%
C-BETA BRANCHED	18.76%	18.02%	18.75%	18.86%	19.80%	19.01%	18.86%	18.19%	18.54%	18.54%
POLARITY	47.21%	49.77%	46.279%	46.08%	51.11%	48.97%	45.98%	53.04%	48.90%	48.90%
SALT BRIDGED	19.08%	20.29%	20.65%	20.63%	22.53%	22.31%	22.26%	28.424%	19.957%	19.95%
HELIX FORMATION:	41.39%	42.01%	43.85%	44.28%	43.87%	44.86%	48.83%	52.47%	42.58%	42.58%
BETA SHEET:	29.73%	29.04%	29.25%	29.18%	20.01%	28.86%	26.83%	24.95%	29.08%	29.08
COIL:	28.85%	28.90%	26.900%	26.55%	26.12%	26.28%	24.35%	22.58%	28.35%	28.35%
POSITIVE CHARGED:	9.80%	10.27%	11.38%	11.50%	12.19%	12.50%	9.85%	3.06%	11.04%	11.04%
NEGATIVE CHARGED:	10.01%	11.00%	11.00%	9.96%	12.55%	11.991%	13.53%	17.30%	11.46%	11.46%

Figure 3 Secondary structure Formation Tendency of Tick Borne Subfamilies

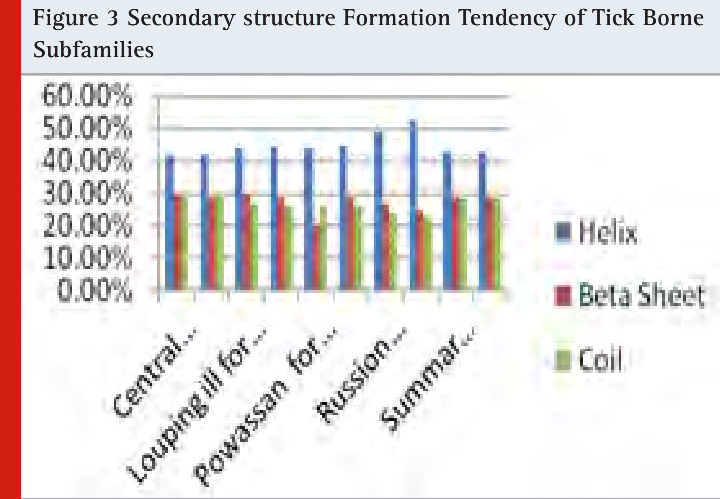
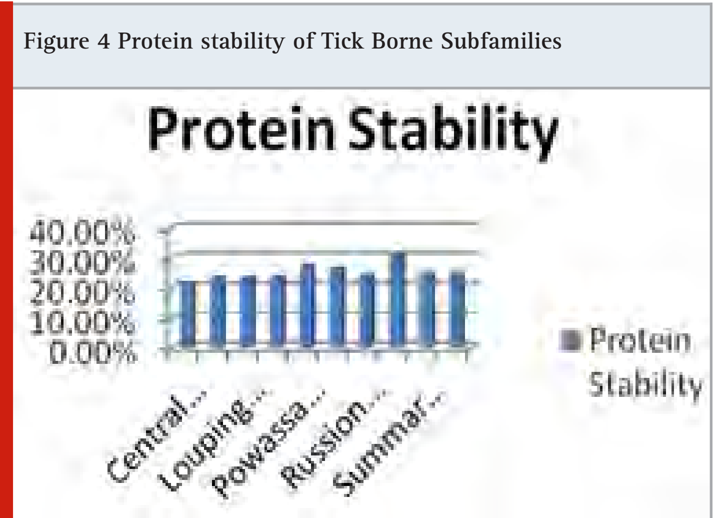


Figure 4 Protein stability of Tick Borne Subfamilies



For 4 frequent Patterns:-

1. {E(Frequent)∩A(Frequent)∩L(Frequent)∩K(Frequent)=>Tendency for Helix Formation}

Tick Borne Subfamilies:-

For 2 Frequent Patterns

1. {A(Frequent)∩L(Frequent)=>Tendency for Helix Formation}
2. {A(Frequent)∩E(Frequent)=>Tendency for Helix Formation}
3. {E(Frequent)∩K(Frequent)=>Tendency for Helix Formation and Protein Solubility}
4. {L(Frequent)∩R(Frequent)=>maintain charge of protein and help in protein stability }
5. {V(Frequent)∩I(Frequent)=>Tendency for Sheet Formation}
6. {G(Frequent)∩S(Frequent)=>Tendency for Coil Formation}

For 3 frequent Patterns:-

1. { E(Frequent)∩A(Frequent)∩L(Frequent)=>Tendency for Helix Formation}

Table 10. Physicochemical Properties of Known Vector Subfamilies				
Physicochemical Properties (Parameters)	Rocio R	Non-R	Rio Bravo R	Non-R
MOLECULAR: WEIGHT	47890.18	48100.777	50921.996	7222.977
EXTENSION COEFFICIENT [assuming all residues of tyr,trp,cys]:	57442.98	58009.14	95937.62	88235.15
ABSORBANCE :	1.406	1.415	1.556	1.551
HYDROPHATICITY [GRAVY]:	-0.175	-0.210	0.321	0.275
ALIPHATIC INDEX:	88.693%	87.751%	110.873%	08.928%
AROMATICITY:	9.205%	9.201%	9.558%	9.106%
PROTEIN STABILITY:	21.997%	22.564%	17.356%	18.189%
C-BETA BRANCHED:	18.433%	18.334%	20.401%	20.638%
POLARITY:	48.404%	49.059%	43.085%	43.980%
SALT BRIDGED:	19.403%	19.775%	16.250%	16.853%
HELIX FORMATION:	41.123%	41.160%	41.653%	40.991%
BETA SHEET:	29.418%	29.392%	31.452%	31.468%
COIL:	29.460%	29.448%	26.896%	27.541%
POSITIVE CHARGED:	10.590%	10.8585%	9.099%	9.249%
NEGATIVE CHARGED:	9.626%	9.850%	6.764%	7.216%

Figure 5. Secondary structure Formation Tendency of Known Vector Subfamilies

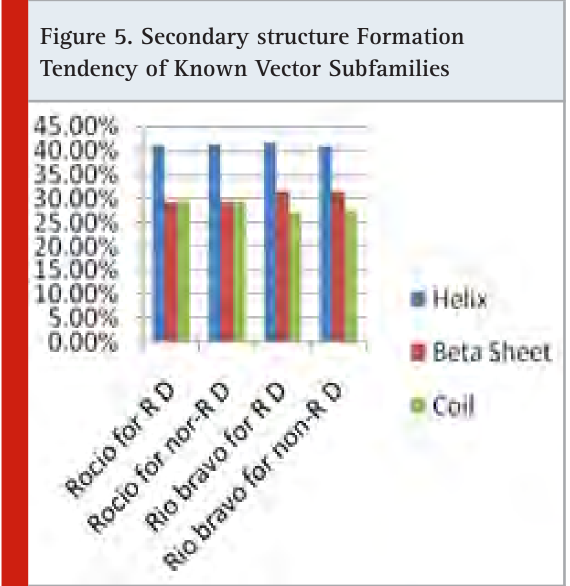
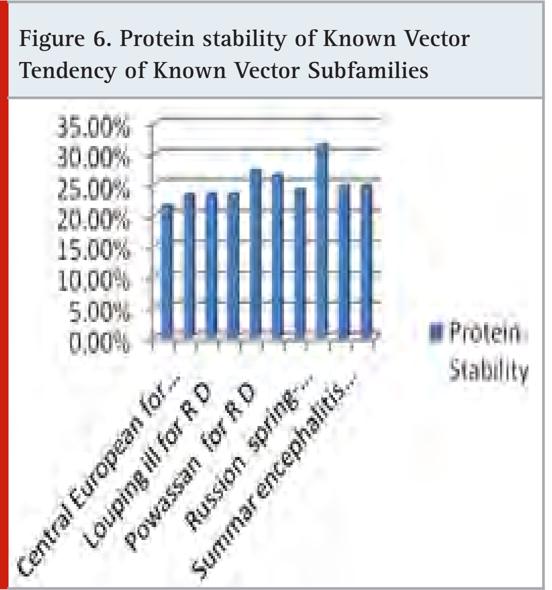


Figure 6. Protein stability of Known Vector Tendency of Known Vector Subfamilies



2. {A(Frequent)∩L(Frequent)∩R(Frequent)=> Tendency for Helix Formation}
3. {E(Frequent)∩K(Frequent)∩R(Frequent)=> Tendency for Helix Formation and Protein Solubility }
4. {I(Frequent)∩T(Frequent)∩V(Frequent)=> Tendency for Sheet Formation}
5. {D(Frequent)∩G(Frequent)∩S(Frequent)=>Tendency for Coil Formation}

For 4 frequent Patterns:-

1. {E(Frequent)∩A(Frequent)∩L(Frequent)∩K(Frequent)=>Tendency for Helix Formation}
2. {E(Frequent)∩A(Frequent)∩L(Frequent)∩R(Frequent)=>Tendency for Helix Formation}

For 5 frequent Patterns:-

1. {E(Frequent)∩A(Frequent)∩L(Frequent)∩K(Frequent)∩R(Frequent)=>Tendency for Helix Formation}

Known Vector Subfamilies:-

For 2 Frequent Patterns:-

1. {A(Frequent)∩L(Frequent)=>Tendency for Helix Formation}
2. {L(Frequent)∩K(Frequent)=>Tendency for Helix Formation}
3. {V(Frequent)∩T(Frequent)=>Tendency for Sheet Formation}
4. {V(Frequent)∩I(Frequent)=>Tendency for Sheet Formation}
5. {G(Frequent)∩S(Frequent)=>Tendency for Coil Formation}
6. {G(Frequent)∩P(Frequent)=>Tendency for Coil Formation}

According to rule 1 for 2 frequent patterns of all subfamilies, it has been observed that amino acids A and L favour helix formation. According to rule 1 for 3 frequent patterns of mosquito and tick borne subfamilies, it has been observed that amino acids E, A and L favour helix formation. According to rule 1 for 4 frequent patterns of mosquito and tick borne subfamilies, it has been observed that amino acids E, A, L and K favour helix formation. According to rule 1 for 5 frequent patterns of tick borne subfamilies, it has been observed that amino acids E, A, L, K and R favour helix formation.Similar interpretation can be inferred by rest of the rules for frequent patterns of all subfamilies in frequent patterns. The above result shows frequent pattern for amino acid in helix formation is maximum than sheet and coil formation.

CONCLUSION

The fuzzy set approach is proposed and employed for prediction of amino acid association patterns in peptide sequences of flavivirus subfamilies. The association rules generated have been used to predict the physiochemical properties and secondary structures as an illustration. The association patterns generated gives the insights of various relationships among amino acids, physiochemical properties and secondary structures. Such models can be developed to generate the information on molecular relationships and mechanisms involved in the disease which could be useful to bio medical scientists for development of methodology for diagnosis and treatment of diseases.

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On the Physical Chemical and Bioremediation Methods for the Removal of Certain Heavy Metals

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ABSTRACT

Metallic entities with quiet a heavier molecular weight have been a part of environment with a subtle existence, but their haphazard use for mankind purposes has amended the natures balance in two of the ways, whether it may be biochemical or geochemical. Outcome from these activity speaks loudly about their excess release in the form nickel, zinc cadmium, copper, lead, and others into the environment. Exposure with them for longer durations and with hugely growing accumulation rates portrays deleterious effects on aquatic forms of life as well as human life forms. Microorganisms with their versatile metabolic activities are somehow indulged in their remediation this has been well studied and implemented till now. The present review is an attempt to scrutinize the capability of microorganisms and as well as plants to remediate the compilation of heavy metals. In accordance to build up a referable literature this review will also show insight to physical and chemical ways. A microorganism when studied at the molecular level gives insight that they accumulate heavy metals and denigrate them at their sites where they are present. The present literature and work review will discuss benevolent strategies to tackle compilations of heavy metals. Microbial life forms which are used for the removal of heavy metals from the water bodies include bacteria, fungi, algae and yeast. Some important antioxidants such as flavonoids, pectin and phytic acid are also used for the elimination of the heavy metals from the human body. The present article is an extensive review that will offer a number of strategies and possible mechanisms for the heavy metals removal both from environment as well as from human body.

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INTRODUCTION

When the density of metallic constitution is 5, i.e., heaviest it belongs to the category of heavy metals. They are important and effective in various practical applications. Sometimes they are part of our diet just because they are very essential to be consumed in trace amounts. Blood iron is usually 0.06-0.6 mg/l, while zinc is 4-8 mg/l , Molybdenum 5-157 micrograms/ Litre, cobalt is 0.09-0.46 µg/ litre, chromium0.05-0.5 µg/ litre, manganese 6.7-10.4 µg/ litre Caroli et al. (1994); Minola et al. (1990). Several other elements called as ultra-trace element are present in very lesser amount like 1µg/g of a 40-290 µg/ litre of lead 1 – 1.14 µg/ litre of Nickle, vanadium 0.1-0.9 µg/ litre, (Nielsen et al. 1984; Rodushkin et al. 1999, Ontarion et al., 2018, Rether and Schuster (2019) and Magda (2019). Few heavy metals which are either metals or semi metallic components (Arsenic) have toxic level as follow. The above MCL standards were given by Babel and Kurniawan, (2003).To eliminate the trace of heavy metals from any sort of components on the basis of suitable conditions can be categorized as.

Physical methods: These methods are being used by numerous researchers to remove the heavy metals. These are primarily efficient and applied on particulate form of metals, distinct particles or metal containing particles, Dermont et al. (2008). The technique consist mechanical screening, hydrodynamic categorization, gravity concentration, magnetic separation, flotation, electrostatic separation, and attrition scrubbing (Dermont et al. (2008). The effectiveness of physical separation is result of different soil characteristics such as particle size distribution, shape of particle , clay content, humidity content , moisture content , density between soil matrix and metal contaminants, heterogeneity of soil matrix, magnetic properties, and hydrophobic properties of particle surface Smith et al.(1995); Williford et al. (2000) Rether and Schuster (2019).

Coagulation and Flocculation: This mechanism is based on zeta potential (α) measurement as the condition to define the electrostatic interaction between pollutants and coagulant – flocculants agents (Maldonado et al. (2014). Coagulation process reduces the net surface charge present on the colloidal particles to stabilize via electrostatic repulsion process Benefield et al. (1982). In flocculation process, the particle size increases frequently due to collisions and interaction of various inorganic polymers formed by addition of organic polymers Tripathy (2006). After the changing of distinct particles into huge particles, they can be filtered easily or removed by the process of straining or floatation. Drawback of this procedure could be production of sludge, chemical applications and application of toxic compounds into solid phase.

Electrochemical Treatments Electrolysis: One of the technologies used for removal of metals is electrolytic recovery. In this process, electric current is passed through an aqueous metal having solution by the help of an indissoluble anode and a cathode plate. The movement of electrons from cathode to anode generates electricity. The heavy metals got precipitated in a weak acidic or neutralized catholyte by forming hydroxides. Electro-deposition, electro-flotation, electro-oxidation and electro-coagulation are some types of electrochemical treatment of water Shim et al. (2014) Magda (2019).

Electro destabilization: Some solids can adsorb both positively and negatively charged ions from an electrolyte solution, and then releases other ions with the same charge in an equal amount into the solution, such as ion exchange resins, in which ions like sodium and hydrogen are interchanged with positively charged ions like nickel, copper and zinc ions present in the solution. In the same way, the negatively charged ions in the resins like hydroxyl and chloride are replaced by negatively charged ions such as chromate, nitrate, cyanide, sulphate and dissolved organic carbon (DOC).

Membrane filtration: This technique is mostly considered and used for the inorganic effluent's treatment. Suspended solids, organic and inorganic components (heavy metals) can be removed by the process. There are so many types of membrane filtration techniques such as nano-filtration, reverse filtration and ultra filtration

can be used depending upon the size of the particles for the elimination of heavy metals from wastewater. In ultrafiltration technique, heavy metals, suspended solids and macromolecules can be filtered depending on the pore size and (5-20 micrometres) and molecular weight of the separating compounds (1000 – 100,000 Da). Ultra filtration is capable of removing more than the 90% of metals concentration ranging from 10 to 112mg/L at PH from 5 to 9.5 at 2.5 Barr of pressure. Ultra filtration have some advantages due to its high packing density such as lower driving force and little space requirement.

Polymer- supported ultrafiltration (PSU): In these techniques, water soluble polymeric ligands are bounded to metal ions, which form macromolecular complexes. They produce a free targeted metal ions effluent Rehter et al. (2003). The PSU technology is advantageous as it requires low energy in ultrafiltration, the reaction kinetics is very fast and thus is higher selectivity of separation of selective bonding agents in aq. solution.

Complexation: Ultrafiltration is another similar technique, that can be used, which is also based on ion exchange and precipitation. In this method, the water soluble metal- binding polymers are combined with ultra-filtration (UF) to filter heavy metals from a solution Petrov and Nenov et al. (2009). Electro dialysis(ED) is a technique in which the solution containing ionized species are passed via an ion exchange membrane by action of an electric potential. These membranes are thin plastic sheets and have either anionic or cationic characteristics. When the solution (having ionic species) is passed through the cell components, the anions are attracted towards cathode, crossing the cation-exchange and anion exchange membranes. Its disadvantages are membranes replacement and the corrosion process Kumiavan et al. (2006). Use of membranes that have higher ion exchange capacity, yields better cell performance. The effect of flow rate, voltage and temperature were studied at different concentrations, using two types of commercial membrane, a laboratory and ED cell, for removal of lead Mohammadi et al. (2004) Magda (2019).

Chemical method of bioremediation: Chemical precipitation: In this process, the pH pf the effluent is increased by using caustic soda (NaOH) or (MgO) and lime (CaO) for precipitating and so, inactivates the heavy metals by changing them to hydroxides Esmaelie et al. (2009). To remove the heavy metal ions from their

Table 1: The MCL *standards for the most hazardous heavy metals*

Heavy metals	Toxicities	MCL(mg/l)
Arsenic	Skin manifestations , Visceral Cancers ,Vascular diseases	0.050
Cadmium	Kidney damage , renal disorder, human Carcinogen	0.01
Chromium	Headache, diarrhoea , nausea, vomiting, carcinogenic	0.05
Copper	Liver damage, Wilson disease, insomnia	0.25
Nickel	Dermatitis , nausea, chronic asthama,human carcinogen	0.20
Zinc	Depression, Lethargy, neurological signs and increased	0.80
Lead	Damage the fetal brain, diseases of the kidneys, circulatory system and nervous system	0.0060
Mercury	Rheumatoid arthritis and diseases of the kidneys circulatory systems and nervous systems	0.00003

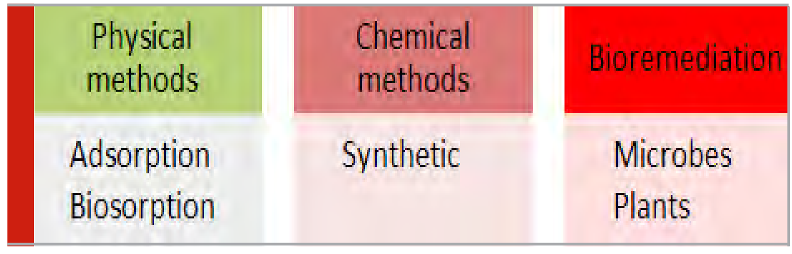
aqueous solution, magnesium oxide was found as a better absorber, at low pH, Asadzadeh et al. (2018). Various coagulants like magnesia, caustic soda, lime, cationic poly electrolyte (CPE) and their combinations were applied to choose the suitable one having most appropriate removal efficiency. In removing the metals, magnesium oxide is found as very effectual even when used small doses.It started precipitating the metals by adsorbing them, before the pH was increased. Situation differs in CaO and NaOH, as they are pH dependent. This proves the MgO to be but precipitator of iron and cobalt.

To calculate the removal efficiency of the metals from its solution :(Ci- C) / Ci * 100%, Where, Ci is the initial concentration of metals, before the addition of coagulant and C is the final concentration of metals, after the addition of the coagulant.In the case pf chromium metals, the CaO reacts with Cr (III) and gives out CaCrO4, which is soluble in water have, the Cr (III) remains in the solution. Whereas MgO reacts with Cr (III) to form $MgCrO_4$, which precipitates in solution (Faon 2006). The precipitation can start with even a little close of MgO. But no adsorption takes place in the case of CaO and NaOH, as they have soluble in water (High TDS) and depends totally on increasing pH attained by chemicals (hydroxide precipitation).

Mechanism of Bioremediation: Micro – organisms are ubiquitous that lies mostly in heavy metal contaminated soil and can easily change heavy metals into non-toxic forms. The organic wastes are converted into mineral end products such as water and CO2 or as metabolic intermediates which can be later used as primary substrates for growth of cell microbes, can produce degradative enzymes for the target pollutants as well as resistance to relevant heavy metals, so they are two- way protected. Many types of bioremediation techniques are known, these includes metal – microbe interactions, bio mineralisation, bio sorption bioaccumulation bioleaching and biotransformation, (Magda 2019). Microbes have a unique property to consume the heavy metals and produce some of their metabolites using this as a catalyst, in order to grow and develop properly. Their capabilities vary from dissolving metals to oxidising transition metals. Various methods are used by microbes to restore the environment such as immobilizing, binding, oxidizing, transformation and volatizing.

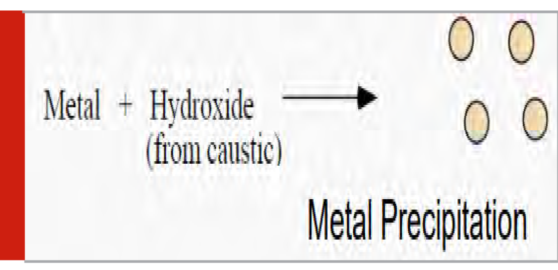
By the application of microbes and controlling their growth and activity at the contaminated sites, and by controlling their metabolic and response to environmental changes, one can succeed in bioremediation of a derived location. Their plasma membrane may be disrupted by certain defence mechanisms like forming outer cell membrane protective materials, other hydrophobic or solvent efflux pumps, cells can survive Sikkena et al. (1995). Many bacteria are resistant to As, Cr and Cd as they have plasmid encoded and energy dependent metal efflux systems consisting of ATPase and chemiosmosis ion/proton pumps Roane and Pepper (2000) Ontarion et al 2018).

Bioremediation by adsorpztion: Microbes have some binding sites in their cellular structure, which can bisorb the heavy metals by using energy. In bacterial cell walls, the extracellular polymeric substances have major effects on acid base properties and metal adsorption, (Guine (2006). EPS or extracellular polymeric substances can compound heavy metals through different mechanism, that are proton exchange and micro precipitation metals, (Guine 2006; Farg et al. 2010).Studies have expressed the proton and adsorbed metals on bacterial cells and cells without EPS to find the importance



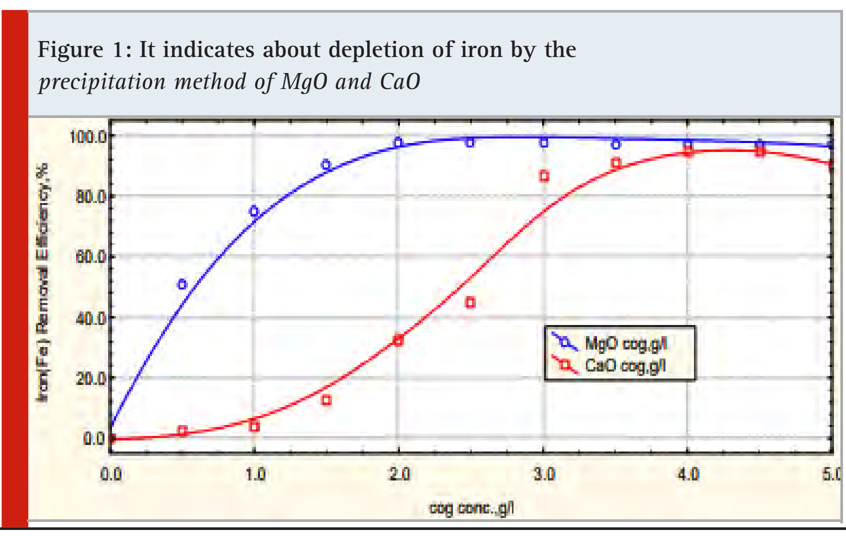
of EPS molecule in removal of metals, (Fang et al. 2011).The era of researches in the sector of bioremediation has not yet got to an end because there are still chances of developments in the genetic composition of microbes. The possible framework for the development of microbe to remove heavy metals has still been under progress,(Gan et al. (2009, Haritash and Kaushik 2009; Onwubuya et al. 2009; Carter et al., 2006; Kinya et al. 1996 Magda 2019) .

Bioremediation by Physio-Bio-Chemical Mechanism: Biosorption is the process which consist higher affinity of a bio sorbent towards sorbate (metal ions), continues till equilibrium is attained between the two components, (Das et al. 2008). Saccharomyces cerevisiae acts as a bio sorbent and remove the Zn (II) and Cd (II) through the ion exchange mechanism Chen and Wang (2007) Talo et al. (2009). Cunninghamella elegans emerged as a perfect sorbent in against of heavy metals released by textile wastewater. Heavy metal degradation involves energy for possessing metabolic cycle in a cell. The combined active and passive modes of toxic metal bioremediation can be called bioaccumulation Brierly (1990). Fungi have emerged as potential biocatalysts to access heavy metals and transform them into less toxic compounds Pinedo-Rivella, (2009). Some fungi such as Klebsiella oxytoca, Allescheriella sp., Stachybotrys sp., Phlebia sp. Pleurotus pulmonarius, and Botryosphaeria rhodina have metal binding potential Annibale et al. (2007). Pb (II) contaminated soils can be biodegraded by fungal species like Aspergillus parasitica and Cephalosporium aphidicola with bio sorption process Tunali et al. (2006); Akar et al. (2007). Hg resistant fungi (Hymenoscyphus ericae, Neocosmospora vasinfecta and Verticillum terrestre) were able to bio transform Hg (II) state to a nontoxic state Kellu et al. (2006). Many of the contaminants are hydrophobic, and these substances appear to be taken up by microbes through the secretion of some biosurfactant and direct cell-contaminant association Lajszner et al. (2018). Biosurfactants form stronger ionic bonds with metals and form complexes before being desorbed from soil matrix to water phase due to low interfacial tension Jhavasi et al. (2011).



Molecular Mechanisms Involved in Bioremediation Process: Various mechanisms involved in the removal of heavy metals by microorganisms are known. In a genetically engineered bacterium Deinococcus geothemalis, Hg reduction has been reported at high temperatures due to the expression of mer operon from E. coli coded for Hg2+ reduction. Mercury resistant bacteria Cupriavidus metallidurans strain MSR33 was

Figure 1: It indicates about depletion of iron by the *precipitation method of MgO and CaO*



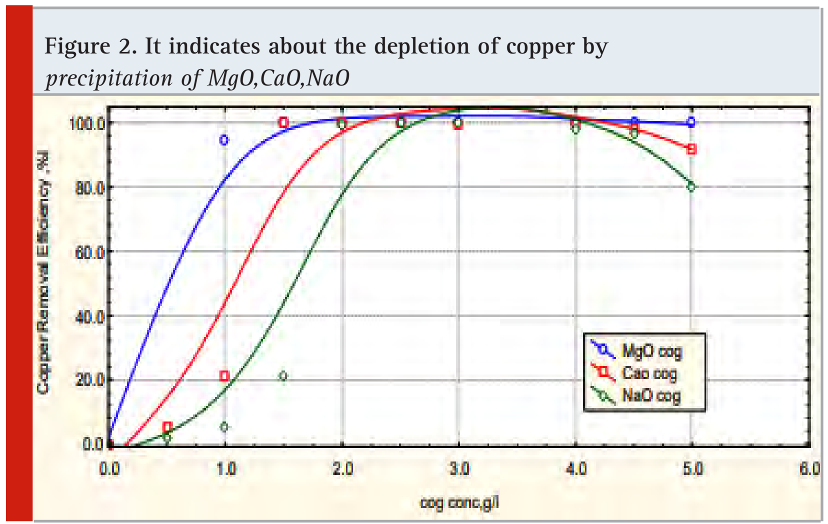


Figure 2. It indicates about the depletion of copper by *precipitation of MgO,CaO,NaO*

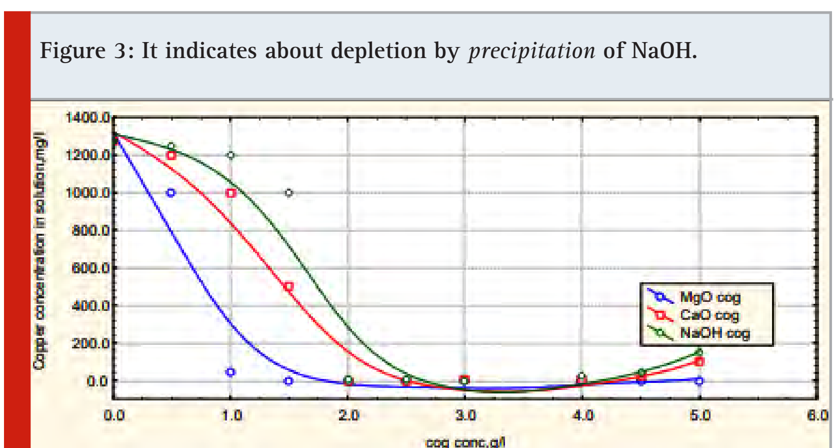


Figure 3: It indicates about depletion by *precipitation* of NaOH.

modified genetically by introducing a plasmid that provided genes (merB and merG) regulating Hg biodegradation along with the synthesis of organ mercurial lyase protein (MerB) and mercuric reductase (MerA) [. Modification of Pseudomonas strain with the pMR68 plasmid with novel genes (mer) made that strain resistant to mercury, (Magda 2019). Two different mechanisms for Hg degradation by bacteria (Klebsiella pneumonia M426) are mercury volatilization by reduction of Hg (II) to Hg (0) and mercury precipitation as insoluble Hg due to volatile thiol (H2S). Genetic engineering of Deinococcus radiodurans (radiation resistant bacterium) which naturally reduces Cr (IV) to Cr (III) has been done for complete toluene (fuel hydrocarbon) degradation by cloned genes of tod and xyl operons of Pseudomonas putida. Microbial metabolites like metal bound coenzymes and siderophores mainly involved the degradation pathway, (Dixit et al. 2015 and Magda (2019).

CONCLUSION

Since, the impact of bioremediation at present or in future is not well known, it can be better alternately compared to chemical and physical methods. Meanwhile, better technical implementations, continuous surveillance, continuous developmental methodologies are necessities .Bioremediation has great advantages as it is more effective in not only destruction but also the complete removal of remaining debris. The filtration techniques are safe to handle and the microorganisms are present naturally as well as can be easily cultured Despite being

better, the studies have shown that degradation of most of the metals are carried out completely. The advantages include easy handling, cost effective, more natural, completely destroys wide variety of contaminants. Also, there is very lesser chance of future liability related to treatments. It does not disrupt the normal activities. The limitations include the limited effectiveness of bioremediation on some organic contaminants. Also, the microbes are more or less affected by the various environmental conditions like pH, temperature, etc. The bioremediation process is capable of removing the contaminants that made the approach cost effective and time saving; there are some studied indigenous organisms which are worth exploiting for the eco-friendly management for the treatment of effluents containing the multiple contaminants. Bioremediation with its certain pros and less cons have been explored and shall be explored with the change in chronology.

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Applications of Feather Keratin Hydrolysate in Divergent Fields

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ABSTRACT
Over the past decades, the feather waste degradation is one of the emerging problems due to the enormous wastes generated from poultry farms. In addition to poultry farms, the leather and wool industries are frequently producing million tons of keratin waste that have a negative impact on the environment. It emphasizes the critical need for research around the world on feather keratin hydrolysates. Therefore, nowadays the feather keratin hydrolysis product plays a significant role in the field of environmental research. Keratin is a tough protein and it is insoluble in nature and not easily degradable due to its chemical structure and the presence of various protease based enzymes. Thus, it creates inherent problems to the overall environment making it an ideal reason for the conversion of feather keratin into various protein hydrolysates which can be reusable. To avoid this issue, the feather keratin hydrolysates have been employed in divergent sectors such as chemical, cosmetic, pharmaceutical, agricultural and biomedical sector. In this review, we have explained the role of keratin in the production of biogas, plant promoting growth hormone, removal of hazardous industrial effluents, wound healing and various other biomedical applications.

KEY WORDS: KERATIN; FEATHER WASTE; DEGRADATION, REUSE.

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INTRODUCTION

Keratin protein has recently engrossed extensive interest owing to its ability to develop by-products using sustainable and renewable sources. It exists in various kinds of sources such as nails, wool, horn-hoof, hairs and feathers. Keratin is a major component of the discarded keratin, which belongs to the scleroprotein group and is made up of a chain of small amino acids with 10,500 Da molecular weight. The nature of this protein is greatly resistant to physiological, biological and chemical agents. It is insoluble in water due to the chemical structure of hydrogen bond, hydrophobic interaction and cross-linked disulfide bonds. In specific, the chicken meat processing industry is at a great focus towards sustainable market growth all over the world. Based on USA Foreign Agricultural Service post reports, consumption of chicken was about 59kg in US; 48 kg in Saudi Arabia, 67.1 kg in Hong Kong and 35.4 kg in Canada as a total domestic per capita (USDA Foreign Agriculture Service, 2014 Sharma et al., (2017). Inappropriately, the feathers thrown out are in low demand and most of them burned, landfilled or converted into feather meal and fed to livestock or used as fertilizers. Discarded feathers cause major impend to human health diseases like fowl cholera and chlorosis.

Every year, about 4 million tons of chicken feathers were generated by poultry processing industries (Xia et al., 2012).The disposable of chicken feathers in the environments causes pollution issues. In India, million tones of chicken feather wastes are generated every year from the poultry processing industries causing significant solid waste problems. The chicken feather does not degrade reluctantly due to the existence of β-Keratin. The β-Keratin is the chief constituent which exists in the chicken feathers. The chicken feather mainly comprises of 91% keratin, 1% fat and 8% of water (Shanmugasundaram et al., 2018). It is tough to degrade even by the existence of proteases such as pepsin, trypsin and papain (Paul et al., 2013). Habitually, the disposal of these feathers is followed by few methods like incineration, alkali hydrolysis, steam pressure cooking and waste disposal sites. However, all of the above such methods not only make waste of highly rich amino acids present in the keratin but also cause production of greenhouse gases utilizing enormous amounts of energy (Xia et al., 2012, Paul et al., 2013 Chen et al 2017).To circumvent these issues, keratin is regenerated into another form by chemical methods that may provide an alternative resolution. The cross- linked disulfide bonds are converted into non-cross linkage forms through oxidation and reduction methods. In reduction process, reductive agents such as sodium metabisulfite and mercaptoethanol are used to cleave the disulfide bonds into cysteine thiols, (Yang, Yao, & Wang, 2018). The keratin can be hydrolyzed into keratin by the oxidation process, oxidative reagents such as peracetic acid are used to convert the disulfide bonds into cysteic acids whereby, the oxidized keratin is referred to as keratose (Yang et al., 2018). The feathers can also be biologically degraded by the action of microbial keratinase. The presence of disulfide bonds in keratin is reduced using the enzyme disulfide reductase due to the action of proteolytic keratinases that degrades the keratin into the byproducts of oligomeric and monomeric polymers (Paul et al., 2013).

The enzyme keratinase can also be generated by a few microorganisms such as *Bacillus sp*, Rhizomucor, *Aspergillus, Absidia, Tricophyton rubrum, Tricophyton mentagrophytes, Tricophyton gallinae, Microsporum gypseum, Microsporum canis, Streptomyces pactum* and *Streptomyces albus* (Paul et al., 2013). The keratin which exists in the chicken feather have been degraded by various microorganisms such as thermophilic *Bacillus species* P-001A, *Fervidobacterium pennavorans, Thermoactinomyces candidus, Pseudomonas aeruginosa, Aspergillus niger, Bacillus licheniformis, Microsporum fulvum* IBRL SD3 and *Streptomyces pactum* DSM40530, (Santha et al., 2019). The high quality keratins films are fabricated for the biomedical applications. The keratin obtained from the chicken feather can also act as a drug delivery system for the regeneration of bone. Typically, the biomaterials synthesized from the keratin were used to generate the drug delivery system and medical devices. The biomaterials are mainly utilized for the tissue regeneration applications such as

cell proliferation, biocompatibility, promotion of cell growth etc. The keratin exhibits a good biocompatibility among the living organisms was proved by the clinical trials on animal models. Hence, the keratins were utilized for divergent biomedical applications. The keratin protein was also utilized for the various productions such as animal feed, keratin films, biodiesel, biogas and bioplastics. The combination of keratin and biopolymer viz gelatin, chitosan and alginate were blended to use for the development of 2D and 3D nanofibrous scaffold for the divergent applications such as skin regeneration, bone tissue engineering, biomedical, wound healing and cartilage tissue engineering (Shanmugasundaram et al., 2018). Fundamentally, these feather wastes are used as feed production for animals and are not economically feasible and its high cost. Nowadays, the utilization of such by-products of animal feed for animal production is also highly restricted. Therefore, the anaerobic digester is an alternative method due to it has material recovery and energy production (Forgács, Alinezhad, Mirabdollah, Feuk-Lagerstedt, & Horváth, 2011) (Xia et al., 2012). Exclusively, the keratin extracted from chicken feathers comprises of beta sheets and alpha helices and loops. It exhibits high concentration of nonessential amino acids like alanine, cysteine, glycine, serine, and valine even as essential amino acids such as tryptophan, methionine and lysine were exhibits at low concentration (Sharma et al., 2017) (Onifade,1998). Based on those treasured details, keratin is now widely employed in a variety of industries akin to medical, cosmetic, pharmaceutical and biotechnological industry (Sharma et al., 2017). The major keratinase productions and characterizations are given in Figure 1 & 2.

Figure 1. Process of keratin hydrolysate production

Collection of samples from different feather dumping sites

Used different media for the isolation of keratin degrading strain

Isolated strain were characterized by morphological, physiological, molecular, purification and biochemical methods

Effective strain from isolation were undergone for following process

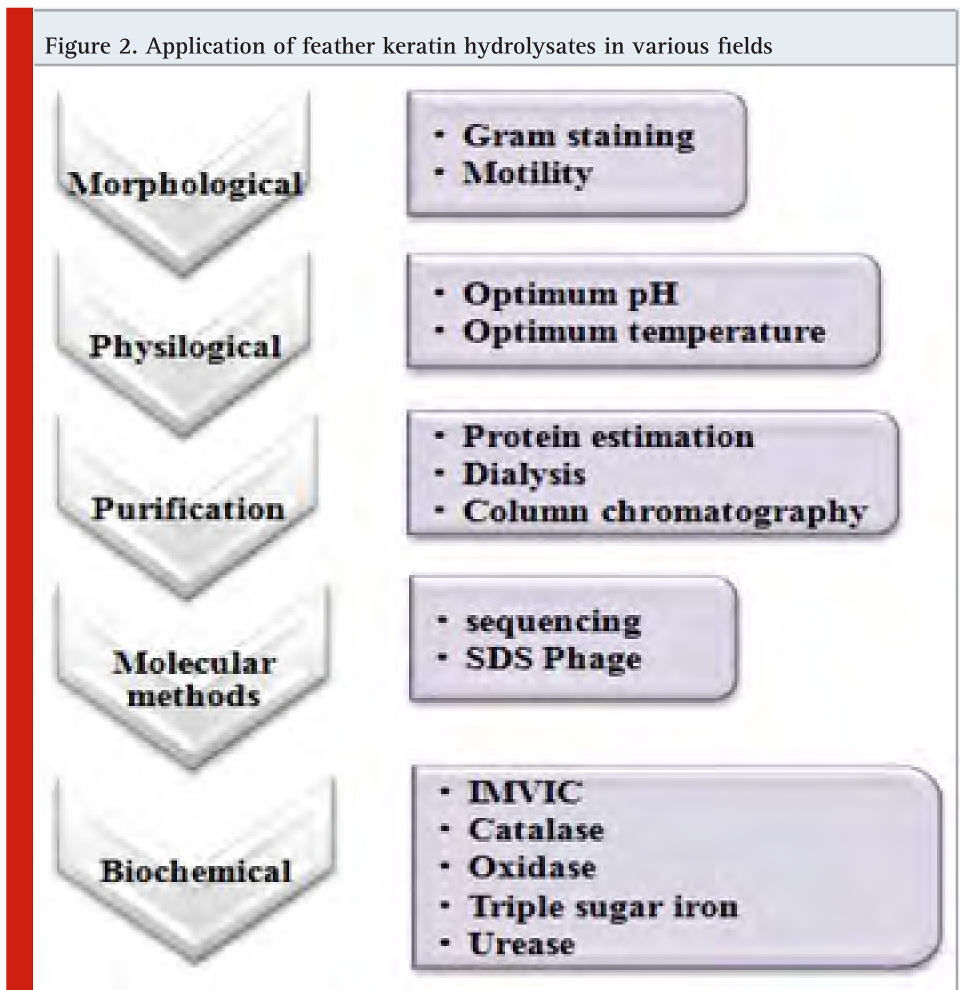
Keratin in Biogas production: Keratin-rich chicken feather waste plays a dynamic role in the biogas production using a two-stage system. This stage comprises of two steps involved in the biogas production. Firstly, the pretreatment step was carried out proceeding to the biogas production. In this pretreatment step, the recombinant strains were employed to degrade the waste chicken feathers. Secondly, the acquired divergent feather hydrolysates following by various pretreatments were implemented for the biogas production. Vidmar and Vodvnik (2019) published an article according to regulation (EC) 1774/2002 of the European parliament, the third category of animal products with explicate meanings: The product obtained from animals, was not proposed for human consumption as it should not spread diseases to animals and humans.

The animal processing waste can be used as substrate for bioenergy and value added products. In older methods, keratin treatments were done by alkaline and acidic hydrolysis, high pressure and high temperature to avoid contamination. This requisite recombinant strain, *B.megaterium* might completely degrade the keratin-rich feather in prior to biogas production, which resulted in the highest yield (80%) of methane. In disagreement, the native keratinase producing *B.licheniformis* could yield about 66% of methane. From this, it has been clearly emphasized that the recombinant *B.megaterium* was more efficient in methane yield than the non-recombinant *B.licheniformis.* This is owing to the existence of xylose inducible

promoter which controls the production of keratinase in the recombinant strain. As a result of this, the efficient keratin degrading enzyme was produced without exhibiting any lag phase during the entire process (Forgács et al., 2011). The high protein content of poultry feather waste is an excellent raw material of bio gas production. Mezes et al., (2019) reported the production of hydrogen sulphide which reached the maximum value. The anaerobic digestion process was achieved by the high bio gas yields as well.

Keratin – a plant promoting growth hormone: The literature states that the *Paenibacillus strain* has an ability to degrade feather and fabricates extracellular enzymes like nitrogenase, keratinase, xylanases and cellulase. Based on the phylogenetic analysis, it reveals that the Paenibacillus were available in various regions such as forest soil, wastewater and desert soil. The strain *Paenibacillus woosongensis* TKB2 was isolated from the soil. It is an effective chicken feather degrading bacterium used for the plant growth-promoting agent. Clain et al., (2019) suggested that protein hdyrolysates having bio stimulating properties are beneficial to plant growth. The result showed that *Trichoderma* isolates were screened for high enzyme activity. The fungal isolates were used as medium with keratin wastes. The output showed improved crop health. Peng and his team (2019) concluded that *Stenotrophomonas maltophilia* had effective feather degraders and to efficiently recycle the chicken feather waste they used S. maltophilia BBE11-1 which showed strongest feather degrading activity. Further,it has been shown that Bacillus licheniformis and arbuscular mycorrhizal fungi play an indispensable role in the field of

Figure 2. Application of feather keratin hydrolysates in various fields



agriculture. Where, B. licheniformis isolated from the chicken feather has the capability to degrade the feather and it also fabricates the indole acetic acid, ammonia and solubilize tricalcium phosphates. Moreover, the findings from pot experiments have revealed that faba bean seeds (*Vicia faba L.*) had potential plant growth in both soil inoculated with or without the combination of arbuscular mycorrhizal fungi (AMF) and bacterial feather hydrolysate (BFH) than the non-inoculated plants. It clearly emphasized, the advantage of using AMF and BFH as a biofertilizer in the agricultural fields, (Nafady, Hassan, Abd-Alla, & Bagy, 2018). Keratin: potential in wound healing: Conventional wisdom holds on the application of keratinase enzyme in wound healing, the enzyme extracts obtained from the chicken feathers were widely utilized for the treatment of wound healing (Wang et al., 2017). These extracted keratins were used in invivo biocompatibility tests to treat the wound, where it provides a moist environment for the wound due to its high water content.

It also has an ability to swell in order to absorb tissue exudates and existence of porous structure which supports cell adherence, cell proliferation and allow permeating the oxygen. In contrast to human keratin, feather keratin is easier to utilize owing to its dyeing and perming of human hair. Therefore, the hydrogels of feather keratin were developed by means of cross-linking reaction exists in the thiol groups with hydrogen peroxide. The keratin developed from the chicken feather exhibits a similar invivo biocompatibility, wound healing effects and biodegradation rates compared to the human hair keratin. As a result, the hydrogels of feather keratin could be employed for biomedical applications and wound healing (Wang et al., 2017). The keratin-rich feather hydrolysate was merged with three various microbial exopolysaccharides such as agar, alginate and gellan to fabricate a therapeutic dermal patch. The dermal patches consist of different wound healing properties such as cell debridement to ensure the topical surface, antioxidant, antibacterial, antifungal as well as tensile strength, water swelling, biodegradability and porosity were also examined to provide the optimum condition for faster wound healing practice. The green synthesized silver nanoparticles were coated on the developed amalgamation patches to prevent the invasion of microbes in the wound. During the development of dermal patches, papain were used to form the enzymatic cell debridement and the activity was calculated by measuring the reduction rate of benzoyl-L-arginine ethyl ester as well as glucose oxidase was added into the patches to control the level of glycemic for topical applications. To improve the progress of wound healing, trolox was loaded in the production of dermal patches and fibroblast growth factor was used to regenerate the skin layer during the wound healing process. From the above, it has been stated that exopolysaccharides based keratins patches are well fitted as a dressing material for wound healing due to its several benefits (Nayak & Gupta, 2017). Yet it has also been reported that the chicken feather keratin hydrolysate was utilized to synthesis non-woven wound dressings through chicken feather keratin and polysaccharides.

The keratin hydrolysates were blended with the polysaccharides such as sodium alginate and chitosan to develop a non-woven wound dressing material such as keratin chitosan (CFK-CS-NW) and keratin sodium alginate (CFK-SA-NW). The fabricated dressing materials might be used for the application of wound healing owing to its physical characteristics like thickness, areal density and air permeability. It also exhibits a potential antibacterial activity in opposition to the Gram-negative bacteria such as Klebsiella pneumoniae, Escherichia coli and Gram-positive bacteria such as Staphylococcus aureus with an effective zone of inhibition. Through biocompatibility test in both invitro and in vivo it concluded that the developed non-woven dressing materials were non-toxic, able to heal the excision wound quickly it also has an ability to support the cell growth. In contrast, the treated chicken feather keratin with chitosan and sodium alginate shows good result than the untreated CFK control group. Hence, the developed feather keratin could be an excellent alternative in the realm of biomedical (Shanmugasundaram et al., 2018). Keratinase enzyme- an effective environmental pollutant removal: Opportunely, the isolated Alcaligenes were employed in various applications such as

the production of keratinase enzyme as well as degradation of chicken feathers and heavy metals. The keratinase enzyme was fabricated up to 10 fold 88.4 U/ml where the highly toxic heavy metals were completely degraded within 36hrs due to the enhanced keratinase activity. Therefore, the heavy metal resistant bacterium such as Alcaligenes has been proved to play an efficient role in the degradation of highly toxic heavy metals and chicken feather wastes for the first time in the field of research. It also been reported that the chicken feather wastes were used in the removal of various heavy metals from the industrial effluents as well as the chicken feather also used as a substrate in the production of keratinase enzyme (Yusuf et al., 2016). Chicken feathers have attractive physiological properties like low density, low thickness 0and warmth retention, high compressibility all of which make it a remarkable fiber than any other natural or engineered filaments. Added advantage of such remarkable fiber reduces the toxicity of arsenite and sulfate. The powdered form of chicken feathers was used as an organic nutrient source for sulfate-reducing bacteria (SRB) as well as arsenic (III) immobilization.

When the powdered chicken feathers were alone act as a carbon source for the sulfate-reducing bacteria, the efficacy in the removal of arsenic (III) and sulfate were decreased. On the other hand, the removal of sulphate and arsenic (III) were enhanced from 30% to 80% in the existence of sodium lactate and powdered chicken feathers. Thus, it has been proved that the powdered chicken feathers incorporated with other electron donors can be utilized as an organic nutrient source for sulfate-reducing bacteria (SRB). The arsenic (III) as well as sulfate was removed from the medium, along with the growth of SRB. The removal of arsenic was interconnected with the reduction of sulfate in the absence of powdered chicken feathers (PCF), however in the existence of PCF; the removal of arsenic (III) is not directly linked with the reduction of sulfate (Costaet al., 2014). The chicken feather waste consists of rich keratinase enzymes were employed in the degradation of crude oil hydrocarbon. The study reveals that the presence of keratinous waste, the removal of crude oil hydrocarbons were increased (57,400 mg l-1) when compared to the absence of keratinous waste (35,600 mg l-1). From the results of chromatographic techniques, it depicts that the incorporation of keratinous wastes were not only removed the aliphatic carbons, however it also changes the pattern of removal of the crude oil hydrocarbons (Cruz-Cumarillo & Avelizapa, 2009).The bioadsorbents are keratin based was developed to treat the hazardous dye effluents. The three dimensional bioadsorbent were fabricated by impregnating with cellulose nanocrystal (CNC) as well as augmented with sponge matrix of keratin to remove the dyes. It was used in waste water treatment due to the existence of various physical characteristics such as large specific surface area, high porosity and abundant adsorption sites in the developed adsorbent. In addition, the keratin augmented adsorbent can be reused for several times owing to its regenerability and reusability. The bio adsorbent shows good result in the fixed bed column by the removal of 80% dyes such as direct red 80 and reactive black 5. Therefore, the chicken feather keratin based bio adsorbent has proven to be a promising technique and exhibits excellent performance in waste water management, hence, it can be of aid to protect the environment (Song, et al 2017).

Keratin in textile industry and leather industry: The keratins extracted from the chicken feather were utilized in textile industries as sizing warp agents in the garment industry. Basically, polyvinyl alcohol has been used earlier as sizing agent in the textile industries. It was non toxic and biodegradable. However, it degrades gradually. To overcome this issue, keratin from chicken feather were used as a sizing agent instead of polyvinyl alcohol. It provides the same strength improvements and cohesion like PVA but it differs by exhibiting the low scratch resistance on garments such as polyester/cotton and polyester. In addition, the keratin exhibits a significant role in the reduction of chemical oxygen demand while comparing to polyvinyl alcohol. Therefore, chicken feather keratin could be an excellent replacement to polyvinyl alcohol as a size warping agent on account of biodegradable, inexpensive and mainly act as an adequate sizing agent (Reddy et al., 2014).The

poultry waste of chicken feather acts as substrate and assist to fabricate the keratinase enzyme production from the keratinolytic bacterium such as Bacillus paralicheniformis MKU3. The synthesized keratinase enzyme was helped out to degrade the feather waste. It can degrade upto 90% of chicken feather wastes. The study also stated that the fabricated keratinase enzyme from the bacterium Bacillus paralicheniformis MUK3 plays an indispensable role in the leather industry owing to it ability in the removal of hair from the sheep skin. It exhibits 100% of results in the dehairing process as well as it aids to avoid the usage of chemicals in the dehairing process (Santha Kalaikumari et al., 2019).

Keratin-a bioactive potential exhibit free radical scavenging as well as anticancer activities: The hydrolysates of chicken feather were acquired by submerged cultivation accompanied by Chryseobacterium sp. Kr6. The antioxidant activity of the supernatant culture was estimated through ABTS radical scavenging method and partially purified using gel-filtration chromatography. Therefore, the bioconversion of chicken feathers with Chryseobacterium sp. Kr6was resulted in protein hydrolysates and consists of potent bioactive peptides with antioxidant properties (Fontoura et al., 2018). Sharma and his team (2017) reported the feather keratin was hydrolyzed by the method of chemical treatment in the alkaline condition. The Keratin based microparticles were examined for the in vitro anticancer activities through sulforhodamine B (SRB) assay in opposition to cell lines such as SK-OV-3, A549 and HeLa. In addition, the cytotoxicity assay was examined towards the cell line by Malin Darby canine kidney (MDCK) and the antioxidant assay was also investigated by the method of DPPH and ABTS. Therefore, the biosynthesized microparticles exhibit a potential anticancer property towards the cell line of SK-OV-3, HeLa and the potent free radical scavenging activity against the ABTS and DPPH method.

CONCLUSION

This review has emphasized about the nature of fibrous keratin with various solicitations which are all eco-friendly methods. As a consequence, there is wide spread interest in utilizing these methods to effectively use the keratinase enzyme. In a way, the magnificent amount of progress has been made in this direction, which actively engaged in method development not only economical but also for better managing of keratinous wastes. Over a last few decades, various pretreatment were performed to enhance the degradability of the keratin containing substances in order to prevent the environmental issues. In contrast, the high-value keratin is one of the important fibrous proteins that have essential value in bio-resource and fertilizer domain. The chicken feather waste can be useful by product of various industries. The hdyrolyzate can also be a nitrogenous fertilizer, bio gas production, animal feed, etc., The hydrolyzate contain rich amino acids for poultry feed development. Hence in future the keratin degraded product can become support a industry level production

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An Identification and Detection Process for Leaves Disease of Wheat Using Advance Machine Learning Techniques

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ABSTRACT

In India, the economic, political, and social stability depends directly as well as indirectly on agricultural productivity. The agricultural product quality and productivity are affected by the different diseases in plants. Therefore, diseases detection in plants is very important in the agriculture field. Leaf disease detection by using different machine learning techniques is a very popular field of study. There have already been many promising outcomes but only a few real-life applications that can make life simpler for the farmer. Machine learning technique becomes the most accurate and precise paradigm for the detection of plant's disease which is helpful to reduce an oversized work of watching in huge farms of crops, and an early stage itself it detects the symptoms of diseases on plant leaves. In this paper, we use a novel approach for detection and identification of leaf diseases by K-means clustering, multi-class SVM, and advanced neural network techniques to process data, on plant disease detection. Gray Level Co-occurrence Matrix (GLCM) is used for feature extraction. The disease classification is done using SVM classifier, and the detection accuracy is improved by optimizing the data using the Advanced Neural Network (NN). The extracted features are fit into the network. This is a great success, demonstrating the feasibility of this approach in plant disease diagnosis and high crop yielding. The aim of our research is solving the problem of detection and preventing diseases of crops. We also demonstrate the model for leaf diseases detection system for future trends of hyperspectral data.

KEY WORDS: ADVANCED NEURAL NETWORK, GRAY LEVEL CO-OCCURRENCE MATRIX, HYPERSPECTRAL DATA, K-MEANS CLUSTERING, SUPPORT VECTOR MACHINE

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INTRODUCTION

In the global economy, agriculture plays a crucial role. With the continued expansion of the human population, the pressure on the agricultural system will increase. Agri-technology and precision farming, now also known as digital agriculture, have emerged as fresh scientific fields using data-intensive methods to boost agricultural productivity while minimizing its effect on the environment. The Advance Machine Learning (ML) method offers high-performance computing to generate fresh possibilities in agricultural operating settings to unravel, quantify, and comprehend data-intensive procedures. ML is defined as the scientific field that gives machines the ability to learn without strict programming, among other definitions. Wheat is the largest humanoid nutrition in the world that can be fed directly from any other crop. Since nutrition is becoming increasingly essential in Asia, where most of the poor people reside (Shuang-Qi et al., 2017). Agricultural productivity is the source of economic growth for developing countries like India.

Chronologically, the primary goal of farming is to provide the country with food and feed it. Plant diseases not only pose a global threat to food security but can also have disastrous consequences for smallholder farmers who depend on healthy crops for their livelihoods. There are more than 80 percent crop production generated by small farmers in the developing countries (UNEP, 2013), and accounts of yield losses of more than 50 percent are prevalent owing to pests and diseases (Abebe et.al.,2017). Also, the biggest proportion of hungry individuals (50%) live in smallholder farming homes (Sanchez et.al, 2005), making smallholder farmers a group especially susceptible to pathogen-derived food supply disruptions. So, these leaf diseases in any forms in crop tend to cause a reduction in quality, yield, and fiscal progression, respectively. Therefore, looking to the current farming arena, instead of watching the crop through the naked eyes by a designated specialist where it does need a lot of efforts to implicate. Hence, as a result, the automation essentiality of leaf disease identification and its management has turned the pen-paper calculation into the reality of high magnitude. Therefore, this work can describe the finding of a solution for minimizing the cost by avoiding manual monitoring and an expert requirement for automatic detection of leaf diseases in a large area (LeCun. et al., 2015).

The plants are vulnerable to various disease-caused illnesses and assaults. The impacts on crops can be characterized by several factors, such as temperature, humidity, dietary excess or loss, bacterial, viral, and fungal illnesses. These diseases can demonstrate different physical characteristics on the leaves together with the plants, such as modifications in form, colors, etc. Due to various comparable patterns, the above modifications are hard to distinguish, making their identification, but the early detection and prevention will prevent the diseases in the entire crop. We will use K-means clustering, multi-class SVM, and advanced neural network techniques on plant disease detection. Gray Level Co-occurrence Matrix (GLCM) is used for feature extraction of plant leaf diseases that affect various plants (Vidyashanakara et al., 2018).

Here we take some of the articles concerning the identification of plant leaf illnesses using different sophisticated methods and some of them shown below. (Fujita et al.,2016) recommended the CNN classifier for cucumber diseases. It uses two datasets for training and validation. In addition to the healthy class, these datasets comprise seven distinct kinds of disease. The first dataset is made up of 7320 targeted images of captured leaves under excellent circumstances. The second dataset comprises of 7520 images captured that are healthy and unhealthy, Lu et al.(2017) defined an automatic diagnostic system for wheat disease based on a supervised deep learning framework that integrates wheat disease identification and disease region. In addition, a fresh wheat disease image dataset, Wheat Disease Database 2017 (WDD, 2017), is being gathered to confirm the system's efficiency. Kamilaris et al.(2018) discussed and studied 40 research papers using deep learning methods that addressed multiple problems in agricultural and food production. Also, examined the specific agricultural problems with the help of the specific

models and frameworks. Ferentinos et al. (2018) explained convolutional neural network models to detect and diagnose crop disease using plain leaf images of healthy and diseased crops using deep learning methods. The training model was carried out using an open database of 87,848 images, consisting of 25 distinct crops in a set of 58 different classes, including healthy plants. Several model architectures were trained with the greatest outcomes reaching 99.53 percent achievement rate when defining the corresponding combination. Kulkarni et al.(2012) described a methodology for early and accurate detection of plant diseases using the artificial neural network (ANN) and various techniques for image processing. Since the suggested method is based on the classification ANN classifier and the extraction function Gabor filter, it delivers better outcomes with a recognition rate of up to 91%.

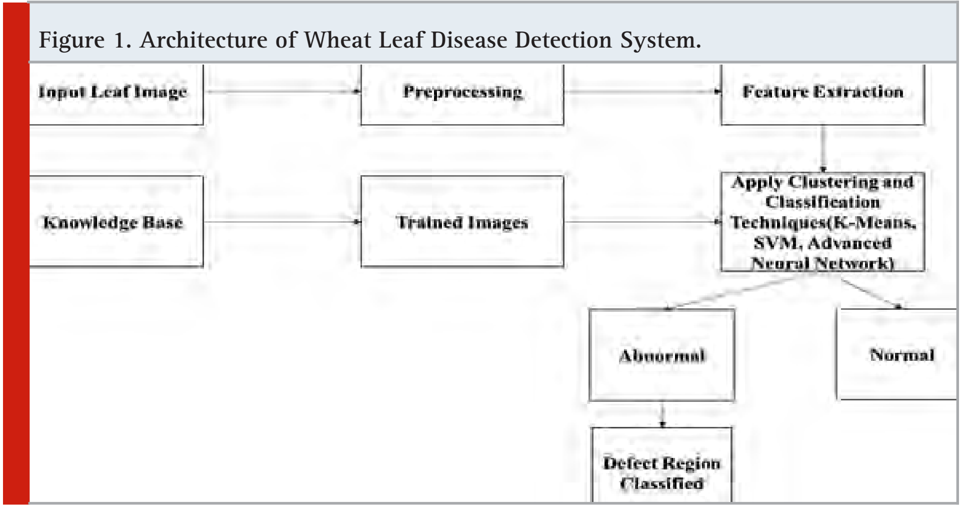
In Malus domestica Bashir et al (2012) provided disease detection using an efficient technique such as K-mean clustering, texture, and color examination. It utilizes the texture and color characteristics that usually appear in ordinary and impacted fields to classify and acknowledge distinct agriculture. Kaundal et al.,(2006) contrasted the results of standard multiple regression, neural network, and support vector machine (SVM). It was discovered that the SVM-based regression approach gives better result. The best description is the association of environmental circumstances with the amount of disease that could be helpful in disease management, (Bannerjee et al.,2018). There are various methods had been already developed to prevent the losses of the leaf disease. However, for many farmers, these methods are unavailable and require thorough knowledge of the domain or a lot of money and resources to be implemented. In this field of research, there are several existing limitations. One of them is that currently available data sets do not include images from real-life situations that have been compiled and labelled. Another limitation is that the existing methods accuracy is not good for the multiple disease in same plant, (Arsenovic et al.,2019). Therefore, in this paper our agenda is to resolve the above limitations by proposing the architectural model for disease detection in wheat leaf by combination of two methods and compare their accuracy.

Leaf Disease Learning Model From Image: We are proposing a Leaf Disease Learning Model from Image data, processing begins in this scheme with data collection in the form of the knowledge base, then training the data, which is used to apply the different machine learning algorithm. Feature extractor steps to be permitted through pre-processing and then identify the illnesses by implementing the various algorithms. Finally, it is possible to classify the defective region from the images. Fig.1 shows our proposed architecture.

MATERIAL AND METHODS

The proposed methodology for disease identification is explained in the following

Figure 1. Architecture of Wheat Leaf Disease Detection System.



sub-sections Dataset Preparation: We can download images from the website https:/ plantvillage.psu.edu/ using the plant and disease names of the keywords. All images can be subsequently categorized into distinct groups. It is an open database with approx. 14,308 Healthy and infected plant leaf pictures were used to train and test the leaf disease detection system. The database used here involves five distinct classes, where each class is described as a couple of leaves of crops and associated disease, while some classes contain healthy crops. There are 30 distinct healthy or diseased plants in these five groups. More than 1/3 of the images accessible (33.3%) were recorded in the field under actual circumstances of agriculture. Fig. 2 shows random samples of images from different wheat classes. The complexity of the images is evident, contributing to several elements such as various leaves and other plant components, irrelevant items, distinct ground textures, shading impacts, etc. Initially, the entire database was split into two datasets, the training set, and the test set, by dividing the 14,308 images randomly so that 75% of them formed the training set, and 25% formed the test set. Thus, 10,731 images were used for the model training, while the remaining 3,577 images were kept testing the model performance in the classification of new, previously ”unseen” images.

Figure 2. Random samples of images from different Wheat Leaf Disease and Healthy Classes (A) Healthy Leaf, (B) Brown Rust Effected Leaf, (C) Black Chaff Effected Leaf, (D) Powdery Mildew Effected Leaf, (E) Yellow Rust Effected Leaf.

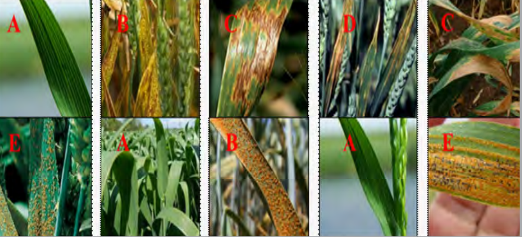
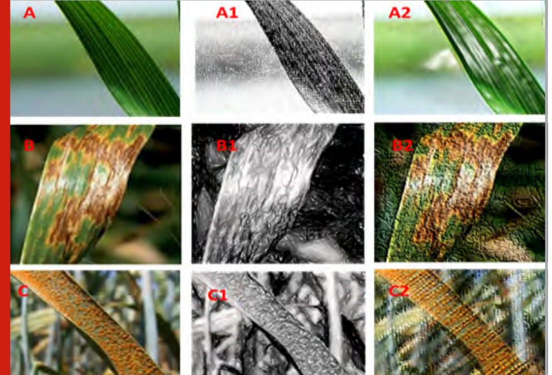


Figure 3. Sample images used in separate experimental settings from the three distinct variants of the Plant Village data set. (A) Leaf1 color, (A1) Leaf1 grayscale, (A2) Leaf1 segmented, (B) Leaf2 color, (B1) Leaf2 grayscale, (B2) Leaf2 segmented, (C) Leaf3 color, (C1) Leaf3 grayscale, (C2) Leaf3 segmented.



The first step for Leaf Disease Detection System initialization. First, take the wheat leaf image that has captured from the digital camera. The color of the input image is primary. The primary color is then transformed as required into a gray-scaled color. Then we experimented with a gray-scaled version of the Plant Village dataset and subsequently run all the tests on a Plant Village dataset version where the leaves were segmented, removed all additional background information that could bring some inherent information. We have evaluated the color, lightness, and saturation aspects of the multiple components of the images. One of the processing measures also enabled us to readily be solved color casts, which occur to be very powerful in some of the dataset subsets. Fig.3 shows for a randomly selected set of leaves with the different versions of the same leaf.

Image preprocessing: Images can be in various formats, quality and resolution in the dataset. For example, images with lower resolution and less than 500px dimensions will not be considered as valid images for the dataset. To extract the necessary data more effectively from the image using pre-processing methods for image resizing and contrast improvement.

Feature Extraction: Machine Learning techniques contain an element at the designing stage. In this stage, specialists propose the hand-made highlights to encourage gaining from precedents. This stage is significant and influences the general execution of the learning framework. Shockingly,

include designing is a manual part of the machine learning technique, and it takes more time (Le Cun. et al., 2015). On the other hand, in an advanced machine learning technique feature extraction is embedded with the learning algorithm where features are extracted in a fully automated way without any intervention by a human expert. Feature extraction plays a vital role in the process of facsimile cataloging. Hence, GLCM could be an effectual and right resourceful technique for statistical parameter extraction based on texture mining (Gebejes et. al, 2013).

The image features include Correlation, Entropy, Variance, Homogeneity, Contrast, Energy, and Mean are computed. The resulted topographies using the monochrome concentration and positioning can be indicated by the association of active dotted particles whose numerical relatives can be estimated in Table1. The graphical representation of the extracted feature is shown in Fig 4.

Training: In this step, train the image of the wheat leaf by using K-means Clustering, Support Vector Machine, and advance neural network for making an image classification model.

Testing: The test set for leaf prediction as healthy/ unhealthy with its disease name will be used in this stage to assess the classifier's output.

Approaches K-Means Based Clustering Method: This stage involves the use of the K-Means algorithm to cluster an image. Using a group of K-classes, it is a very helpful technique for entity recognition (Dubey et al., 2013). It can be done by limiting the square summation distance between

Table 1. Average Extracted Features Vector Using GLCM Method.

S. No.	Contr ast	Corre lation	Energy	Homog eneity	Mean	Standard Deviation	Ent ropy	RMS	Variance	Kurtosis	Skew ness
1	0.520	0.82726	0.81	0.956992	11.42	42.14	1.088	3.194	1523.1	17.921	1.8645
2	0.453	0.88268	0.741	0.942985	18.62	51.96	1.6572	4.679	2206.1	10.582	2.3426
3	1.195	0.76266	0.57	0.931926	25.981	60.49	2.1984	7.044	3429.7	7.2729	1.8866
4	0.6984	0.91896	0.602	0.955868	35.465	71.82	2.391	7.347	4946.8	4.3601	2.3403
5	1.2426	0.76266	0.78	0.939879	12.649	46.03	1.0954	3.338	1823.7	16.636	3.0963
6	1.2304	0.91896	0.376	0.932311	36.484	61.36	3.7752	9.044	3009.9	5.777	2.3511
7	0.7108	0.68088	0.655	0.929282	24.433	58.77	2.0447	4.267	2085.4	7.2405	3.2904
8	0.9014	0.80841	0.576	0.927381	35.799	71.94	3.6071	7.978	3948	5.0127	3.1041
9	1.1222	0.87418	0.698	0.943924	28.194	69.98	2.4453	6.015	3597.8	6.7964	3.0347
10	1.361	0.90097	0.724	0.935854	18.952	55.79	1.9201	5.197	2359.1	11.386	4.1762
11	0.7258	0.8651	0.614	0.925867	23.879	56	2.135	5.346	2258.1	7.4625	3.7641
12	0.4065	0.73879	0.75	0.94786	17.785	56.2	1.5335	4.562	2827.8	12.644	0.8359
13	1.221	0.8602	0.734	0.923988	15.923	48.57	1.4202	4.269	2039.3	11.61158	1.6122
14	0.7435	0.91903	0.757	0.938948	18.945	56.55	1.8782	4.812	2798.3	10.949	2.836
15	0.2743	0.70682	0.823	0.976112	13.225	44.84	2.1005	5.159	1703.5	20.069	1.3342
16	0.2681	0.85746	0.786	0.962688	14.843	47.85	2.0682	4.844	1490.1	16.601	1.3215
17	0.6847	0.91649	0.378	0.906731	59.88	78.53	4.5886	9.207	2859.6	2.1288	4.221
18	1.1217	0.92901	0.48	0.896516	40.152	73.24	3.153	7.711	4661.7	4.0614	3.0734
19	0.5468	0.92833	0.723	0.947347	17.922	49.77	1.6294	4.534	2051.4	10.111	0.4327
20	1.8418	0.87307	0.478	0.883095	45.042	77.61	2.9739	7.068	4540.2	3.109	0.745
21	1.5751	0.84231	0.843	0.903862	47.808	80.98	3.1664	7.936	5354.2	3.0829	1.8645
22	0.8019	0.81905	0.709	0.96402	12.901	46.13	2.0809	5.212	2015.2	19.969	2.3426
23	0.328	0.86446	0.237	0.944129	16.123	44.55	1.6311	2.662	352.88	12.241	1.8866
24	1.4987	0.76745	0.27	0.877284	77.399	84.8	4.791627	11.2647	6629.51	1.5772	2.3406
25	1.346	0.86944	0.458	0.912759	62.05059	74.62	4.5629	10.71	4388.6	2.1224	3.0963
26	0.5789	0.87351	0.789	0.97456	68.999	66.78	4.3245	6.745	345.89	2.0789	2.0098
27	1.673	0.86979	0.399	0.982114	38.987	56.93	1.89789	3.679	661.78	9.0789	1.789
28	0.456	0.93412	0.897	0.911239	47.345	33.78	3.4567	5.8769	2897.9	13.1345	0.3344
29	1.876	0.8569	0.769	0.901987	59.8796	77.84	2.9897	10.78	2345.67	2.45908	5.789
30	1.798	0.789	0.749	0.80796	53.5346	79.1	1.79854	4.8999	3987.9	10.9089	1.0459

the equivalent cluster and an entity to find the thought-provoking fragment of the source image. In K-Means clustering techniques, clusters are determined by pixel groups with the same value current in an image. Practically, this new image processing technique's computational acceleration is very quick and provides a more precise output. The input data set is divided into K set of clusters, and each cluster is represented by a completely variable cluster center. Initially, known values are referred to as seed points and data points are also known as inputs. Only the K-Means clustering method can be used to estimate the distances between the centers, inputs, and allocate inputs to the closest center. Following are the steps for K-means grouping:

Step 1: Image confirmation
Step 2: Image transformation from primary color space to L*a*b* Color Space, which helps in the findings of pictorial transformations that present in the primary **color space.**

Step 3: Here the colors classification can be done in 'a*b*' Space using K-means.

Step 4: From the results, pixel ordering, and labeling can be render using K-means by maintaining a return index to the corresponding each cluster.

Step 5: Afterwards, the original image is partitioned based on its k- number of color cluster.

This process has already been implemented in other leaf images to cluster more than one groups having the respective diseases (Sethy et al., 2017). After the successful implementation K-Means cluster-based segmentation, the percentage of the infected area calculated, and features are extracted.

Classification of Diseases by SVM: In this phase, the classification and comparison for the leaf diseases of the wheat crop have done through by storing the corresponding feature set of values to their respective dataset. Here a fully controlled learning method of classification is used that is none other than support vector machines (SVM) with high dimension spaces, efficient memory, and versatile decision function. Generally, SVM is categorized into two types: Linear SVM and Multiclass SVM. Linear SVM is used to classify two kinds of data set, and multiclass SVM is used to classify more than two kind of data set. So multiclass SVM is used to classify four types of wheat leaf diseases. First, the extracted feature dataset is optimized using the optimization technique, and then multiclass SVM is used for the classification process. The quantity of cataloging is performed by the proportion of high-scale classification gain, Equation (1).

$$\text{Classification Gain (\%)} = \frac{\textit{Number of Correct Classifiacttions}}{\textit{Total Number of Test Images}} * 100 \qquad (1)$$

Advanced Neural Network: There are two significant drawbacks to the machine learning

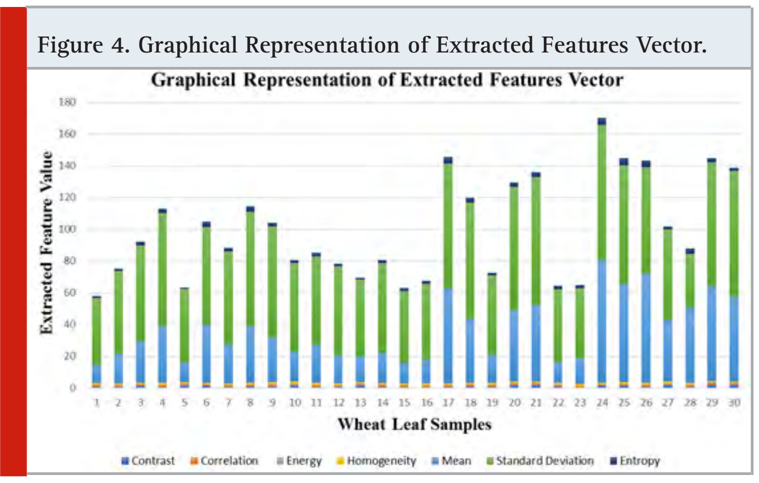


Figure 4. Graphical Representation of Extracted Features Vector.

techniques. First, they are extremely dependent on variables patterns as well as characteristics to be extracted. Second, it is necessary to train classifiers many times before applying to applications in the real world (Zhang et.al., 2015) ANNs (Advanced Neural Networks) are the most promising tools for analyzing hyperspectral data. ANNs ' mechanism is based on the nervous system of humans. ANNs are very helpful for pattern recognition, irrespective of any specific guidelines for recognition. Cui et al. (2018) stated that ANNs need fewer formal statistics and are capable of modeling complicated nonlinear situations. There is an increasing interest in applying ANNs to achieve the greater goal of disease detection in wheat crop using hyperspectral data. ANNs have

Figure 5. Linear Regression between the Mean and Entropy of the Wheat Leaf.

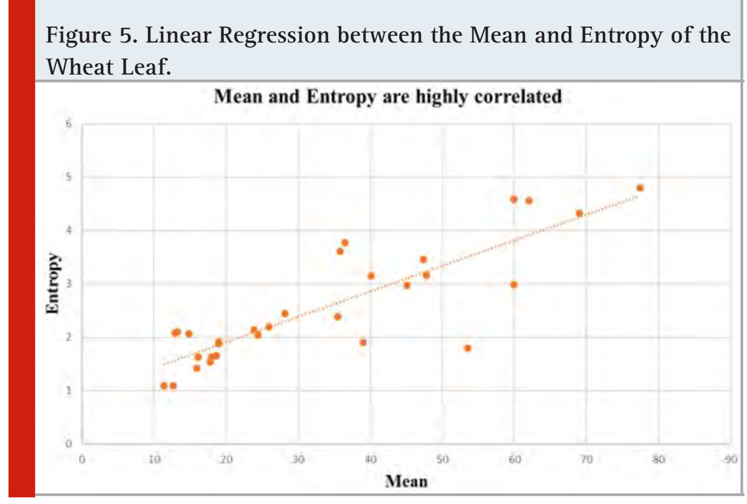


Figure 6. Advanced Neural Network architecture for leaf disease detection through plain and GLCM image.

Figure 7. (A) Apply as an Input Image (B) Black Chaff Disease Detected

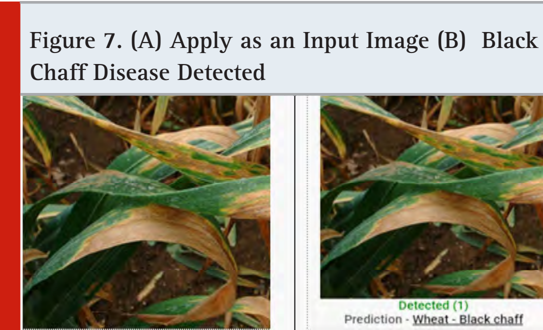
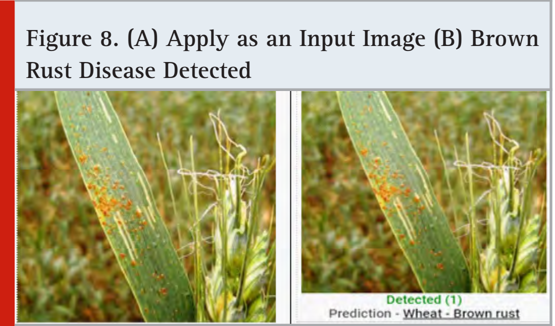


Figure 8. (A) Apply as an Input Image (B) Brown Rust Disease Detected



a higher degree of diagnosing ability than Neural Networks (NNs) (Ettabaa KS, et. al.,2018). As a non-invasive rapid detection method, (Zhu et al.,2017) explored the potential of hyperspectral imaging. The Advanced Neural Networks support the strongest ability to discriminate against plant diseases because they combine the finest sets of trainers for precise classification. It can be used to detect four diseases, namely black chaff, brown rust, powdery mildew, yellow rust. The proposed framework for the advanced neural network represented in Fig. 6. This framework helps to detect leaf diseases accurately and automatically.

RESULTS AND DISCUSSION

There is various type of diseased wheat leaf samples is taken as input. In this paper, four types of wheat leaf diseases are considered i.e. Brown

Figure 9. (A) Apply as an Input Image (B) Powdery Mildew Disease Detected

Figure 10. (A) Apply as an Input Image (B) Yellow Rust Disease Detected

Table 2. Classification results for different diseases for Proposed Method

Leaf Disease	Black Chaff Disease	Brown Rust Disease	Powdery Mildew Disease	Yellow Rust Disease	Accuracy (%)
Black Chaff Disease	30	0	0	0	100
Brown Rust Disease	0	28	0	2	93.33
Powdery Mildew Disease	0	0	30	0	100
Yellow Rust Disease	0	3	0	27	90
	Average Accuracy (%)				95.83

Table 3: Comparison of different classification techniques for different diseases. (a) Combination of the Gray-Level Co-Occurrence Matrix and K- Nearest Neighbor Experimented values, (b) Combination of the Gray-Level Co-Occurrence Matrix and Artificial Neural Network Experimented values, (c) Combination of the Gray-Level Co-Occurrence Matrix and Support Vector Machine Experimented Values.

Leaf Disease	GLCM+ KNN[a]	GLCM+ ANN[b]	GLCM+ SVM[c]
Black Chaff Disease	78.02	84.00	91.11
Brown Rust Disease	80.00	85.50	90.02
Powdery Mildew Disease	75.20	88.08	90.00
Yellow Rust Disease	78.40	85.00	91.01
Average Accuracy (%)	77.90	85.64	90.50

Figure 11. Graphical representation of Classification of different diseases for Proposed Method.

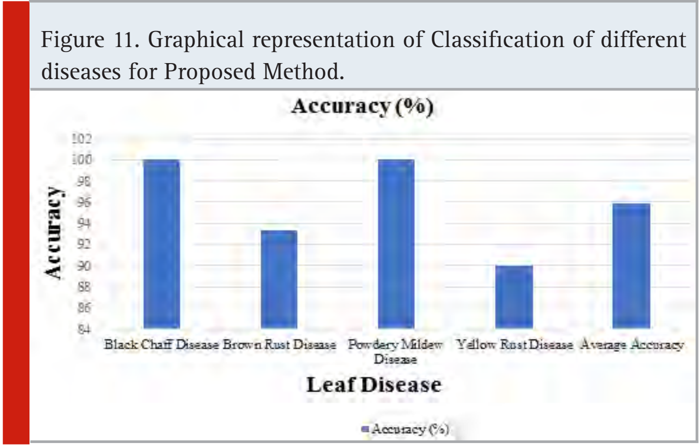


Figure 12. Graphical representation for Comparison of different classification techniques for various Diseases.

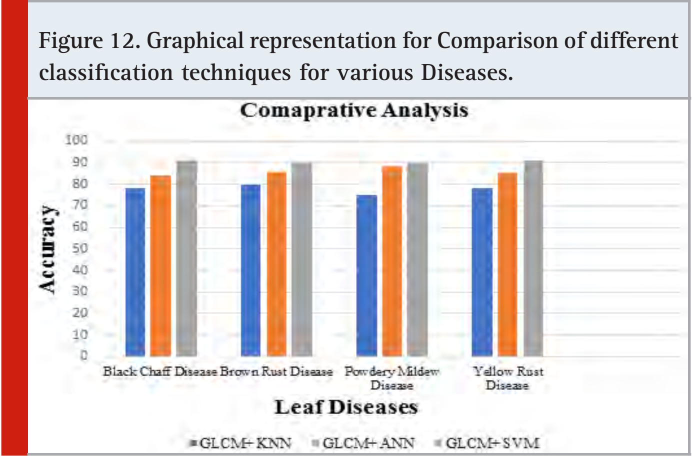
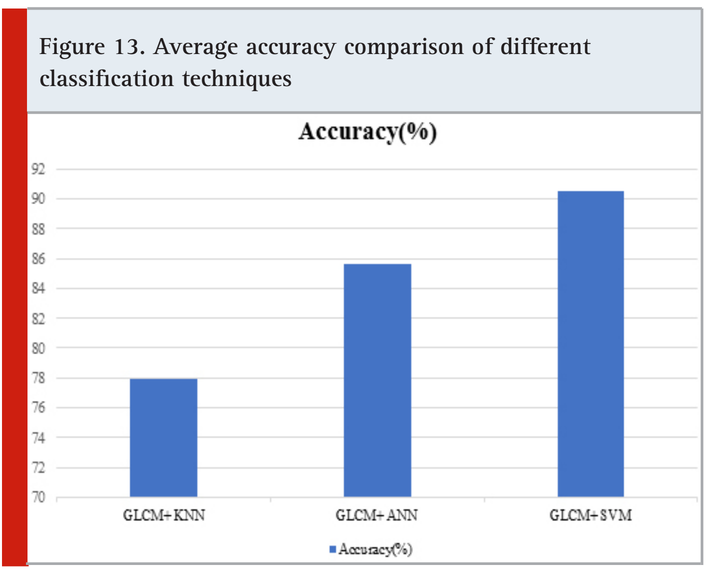


Figure 13. Average accuracy comparison of different classification techniques



Rust, Black Chaff, Powdery Mildew, and Yellow Rust. Fig. 7 shows the input image and output image classified as wheat Black Chaff disease. Fig. 8 shows the input image and output image classified as Brown Rust disease. Fig. 9 shows the input image and output image classified as Powdery Mildew disease. Fig. 10 shows the input image and output image classified as Yellow Rust disease. By the help of this proposed method, the total of 30 sets of wheat crop leaf disease specimens is taken and categorized into four broad categories of diseases which has displayed in Table 2 and Fig. 11. Among 30 number of test samples of Brown Rust Diseased leaf and Yellow Rust Diseased are erroneously classified as Brown and Yellow Spots on the leaf, which imply 93.33% and 90.00 % of accuracy respectively. And the other two categories of diseased leaf i.e. Black Chaff Disease and Powdery Mildew disease are successfully classified with 100% of accuracy. The average accuracy of the classification of the proposed method is 95.83%. In this paper, classification is first done using the K-Nearest Neighborhood (KNN) using K-Mean's grouping with a productivity accuracy of 77.90%.

The finding precision is enhanced by 85.64% using the advanced neural network (ANN). For the next stage, cataloging is done through using SVM ordering process and whose productivity accuracy of 90.50%. Here also we compare with other classification techniques with our proposed method and examine the performance analysis with respect to every four types of diseases which is illustrated in Table 3 and Fig.12. After all, among all classification techniques, the SVM technique result is outperforming, i.e. accuracy of 90.50%, illustrated in Fig. 13.

CONCLUSION

In this paper, we suggest a leaf disease prediction model that can learn from the image datasets and then apply the different methods of machine learning for disease identification of the infected leaf. Protection of crops in organic farming is not an easy matter. Based on K-means clustering, SVM, advanced neural networks, advanced machine learning models were created in our scheme to detect leaf diseases. Our model use images that were captured by different camera systems and gathered from different resources. Our experimental findings and comparisons between distinct techniques with feature extractors showed how our sophisticated machine-learning-based model could effectively identify distinct categories of diseases in distinct crops as well as provide solutions for diseases. The proposed methodology was successfully tested and verified from the execution point of view on various types of wheat leaf diseases such as black chaff, brown rust, powdery mildew, yellow rust. Moreover, it was seen that the best result could be obtained resourcefully using least methodical pains to check the productivity of scheduled techniques. Another perspective of employing this method is that the plant disease can be documented at the beginning or primary stage only. SVM, therefore, proved to be the promising method for differentiating and categorizing wheat leaf diseases with other methods by using extracted feature vector dataset. Collecting a larger number of samples with more variation of the diseased wheat leaf can extend the study. We hope that our suggested scheme will contribute to agricultural research in a provocative manner.

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Comparative Fermentable Sugar Yield from Pretreated Rice Straw by *Trichoderma reseei* and *Aspergillus tamarii* Using Plackett–Burman Method

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ABSTRACT

A study was conducted using Plackett-Burman design to the statistical screening of seven growth medium components viz. glucose, malt, peptone, ammonium sulphate $(NH_4)_2SO_4$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), monopotassium phosphate (KH_2PO_4), and ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) by employing microbial stains Trichoderma reesei MTCC No. 164 and Aspergillus tamarii MTCC No. 8841 on alkali pretreated and autoclaved rice straw for comparative fermentable sugar (viz., xylose and glucose) estimation. All the experimental trials were conducted in triplicate and mean value was recorded for further analysis in identification of significant factor using ANOVA analysis at α value of 0.05 and 95% confidence level. In both of A. tamarii, out of seven growth medium component used, monopotassium phosphate and glucose were found as the significant factor responsible for the release of maximum xylose concentration of 36.03 mg/g whilst ammonium sulphate was identified as a key medium component for the release of the maximum glucose concentration of 29.84 mg/g. In Treesei broth, out of seven growth medium component used magnesium sulphate heptahydrate, glucose and peptone were screened out as significant medium component responsible for the release of maximum xylose concentration of 22.15 mg/g whilst malt, ammonium sulphate and glucose were found responsible for the release of the maximum glucose concentration of 24.31 mg/g. The microbial growth media composition used was found to be more effective for A. tamarii as compared to T.reesei for production of fermentable sugars from alkali pretreated rice straw. Thus the data of the present study establishes optimum valus of selected microbial growth medium parameters responsible for enhanced yiled of fermentable sugar.

KEY WORDS: TRICHODERMA REESEI, ASPERGILLUS TAMARII, MEDIUM COMPONENT, RICE STRAW, PLACKET BURMAN DESIGN, GLUCOSE, XYLOSE.

ARTICLE INFORMATION

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INTRODUCTION

Currently, lignocellulosic biomass is being exploited extensively by many researchers across the globe for developing cleaner and sustainable energy as an alternative to the fossil fuel system (Prasad et al., 2007). Rice is an important food crop in India which is being produced at an annual rate of 106.54 million tonnes of rice and approximately 160 million tonnes of straw with a ratio of 1:1.5 for rice grain produced to straw produced (MNRE, 2017). MNRE, 2017. Energy Generation from Paddy Straw. https://mnre.gov.in › file-manager › akshay-urja › june-2017 (Singh et al., 2008). Due to lack of any suitable utilization system of this huge volume of rice straw farmers generally tend to either burn it in open field or left it in the field as a soil conditioner which ultimately affecting human health severely due to air pollution caused by burning of it and increased methane emission respectively (Singh et al., 2014).

Rice straw may be utilized for producing fuel ethanol since its structural component of the primary cell wall contains cellulose from around 32 to 47%, hemicellulose from 19 to 27% and lignin around 5 to 24% (Garrote et al. 2002; Parameswaran et al. 2010; Zamora and Crispín 1995). The cellulose and hemicellulose is an important constituent of rice straw and can be converted into simple monomeric carbohydrates such as glucose and xylose by effective pretreatment and hydrolysis methods (Sarkar et al. 2012 Cekmecelioglu and Demirci 2018). Pretreatment steps play an important role in liberating lignin and hemicellulose compounds making cellulose and hemicellulose more accessible for enzymatic conversion into fermentable sugars (Jamaldheen et al. 2019; Nigam and Singh 2011). Alkali pretreatment of rice straw has been reported as one of the most efficient method of rice straw pretreatment (Sathendra et al. 2019; Singh et al. 2011). Screening an important medium component affecting the production of fermentable sugar by Placket Buman design methods14 proves to be an efficient way as compared to one factor at the time method. Statistical significance of individual factor may be estimated using placket Burman design methods in a less number of experiments (Singh and Bishnoi 2012 Thi et al. 2018). In the present research, the medium component affecting the production of fermentable sugar was studied using Placket-Barman design method. The individual factors were screened based on the statistical significance of each medium component at alpha value of 0.05 or 95% confidence level.

MATERIAL AND METHODS

Microorganism and Inoculum Preparation: Trichoderma reesei and Aspergillus tamarii, used for the release of glucose and xylose from cellulose and hemicellulose of alkali pretreated rice straw. Both the microbial cultures were procured from Microbial Type Culture Collection Center assigned accession no., MTCC 164 and

Table 1. Assigned concentration of variables at different levels in Plackett–Burman design

S. no.	Medium component	Code	High value (+1) (% w/v)	Lower value (-1) (% w/v)
1	Malt extract	X1	0.75	0.0625
2	Ammonium sulphate	X2	1	0.1
3	Monopotassium phosphate	X3	1.25	0.083
4	Magnesium sulphate heptahydrate	X4	0.075	0.0075
5	Glucose	X5	2.5	0.0416
6	Ferrous sulphate heptahydrate	X6	0.5	0.05
7	Peptone	X7	1	0.25

Table 2. Plackett–Burman design for 7 components with coded values along with observed result for Xylose and Glucose as response

Run	X1	X2	X3	X4	X5	X6	X7	Xylose in A. tamarii broth	Response (mg/g)		
									Xylose in T.reesei broth	Glucose in A. tamarii broth	Glucose in T.reesei broth
R1	1	-1	-1	1	-1	1	1	28.38	18.757	23.12	17.45
R2	1	1	-1	-1	1	-1	1	25.4	19.539	29.05	20.21
R3	1	1	1	-1	-1	1	-1	21.49	16.232	25.34	17.32
R4	-1	1	1	1	-1	-1	1	19.4	16.078	22.46	18.58
R5	1	-1	1	1	1	-1	-1	28.29	22.15	29.68	24.31
R6	-1	1	-1	1	1	1	-1	24.36	18.817	28.89	22.92
R7	-1	-1	1	-1	1	1	1	36.03	19.807	29.84	20.62
R8	-1	-1	-1	-1	-1	-1	-1	18.85	18.054	22.52	20.18

Table 3. Screening of significant medium component affecting fermentable sugar release in *A. tamarii* broth as per Plackett-Burman design

Fermentable sugar	Medium component level (%)	□(H)	□(L)	Difference	Effect	Mean Square	F value	P value	Confidence
Xylose	X1	107.19	103.71	3.48	0.87	1.514	5.447	0.052	94.77
	X2	105.74	105.16	0.58	0.145	0.042	0.151	0.709	29.11
	X3	107.32	103.58	3.74	0.935	1.748	6.291	0.041	95.95
	X4	104.15	106.8	-2.65	-0.663	0.878	3.159	0.119	88.12
	X5	117.46	93.44	24.02	6.005	72.12	259.502	0.007	99.33
	X6	107.19	103.71	3.48	0.87	1.514	5.447	0.052	94.77
	X7	104.47	106.43	-1.96	-0.49	0.48	1.728	0.23	76.99
Glucose	X1	103.56	98.64	4.92	1.23	3.026	1.088	0.332	66.85
	X2	90.65	111.55	-20.9	-5.225	54.601	19.641	0.003	99.7
	X3	105.21	96.99	8.22	2.055	8.446	3.038	0.125	87.51
	X4	100.43	101.77	-1.34	-0.335	0.224	0.081	0.785	21.55
	X5	114.08	88.12	25.96	6.49	84.24	30.302	0.079	92.09
	X6	110.26	91.94	18.32	4.58	41.953	2.205	0.181	81.88
	X7	109.21	92.99	16.22	4.055	32.886	11.83	0.08	91.99

MTCC 8841, at Chandigarh, India. The obtained cultures were maintained on potato dextrose agar (PDA) slants at 4°C before inoculating culture with the substrate. For preparing inoculum Trichoderma reesei and Aspergillus tamarii were sub-cultured using autoclaved Malt Extract Agar medium and Czapek Yeast Extract Agar medium at 121°C at 15 psi for 20 minutes, respectively. The inoculated slant cultures were incubated at 28°C for 7 days after that the inoculum was obtained by adding 5 ml of sterile water to slant cultures and scrubbing the surface of slant using sterile inoculating loop wire (Kadowaki etal. 1997 Wanger et al. 2018). Substrate Preparation and Alkali Pretreatment: The rice straw was collected from the local farmer's field near to Greater Noida, U.P. The obtained rice straw was cut into pieces of size 1 cm long after that the substrate was washed with tap water to remove any dust particle attached with rice straw. The substrate was kept

Table 4. Screening of significant medium component affecting fermentable sugar release in *T.reeseibroth* as per Plackett-Burman design

Fermentable sugar	Medium component	□(H)	□(L)	Difference	Effect	Mean Square	F value	P value	Confidence level (%)
Xylose	X1	79.29	82.3	-3.01	-0.753	1.133	1.179	0.314	68.64
	X2	79.03	82.56	-3.53	-0.883	1.558	1.621	0.244	75.64
	X3	80.83	80.76	0.07	0.018	0.001	0.001	0.981	1.94
	X4	183.26	78.33	104.93	26.233	1376.288	1432.1	0	100
	X5	88.06	73.53	14.53	3.633	26.39	27.461	0.001	99.88
	X6	78.31	83.28	-4.97	-1.243	3.088	3.213	0.116	88.38
	X7	76.86	84.64	-7.78	-1.945	7.566	7.873	0.026	97.37
Glucose	X1	76.68	72.76	3.92	0.981	1.923	7.482	0.033	96.71
	X2	70.67	78.77	-8.1	-2.026	8.205	31.927	0.001	99.92
	X3	74.27	75.17	-0.9	-0.225	0.101	0.394	0.55	44.98
	X4	75.8	73.63	2.17	0.543	0.589	2.29	0.174	82.6
	X5	80.31	69.12	11.19	2.798	15.658	60.925	0	99.99
	X6	73.61	75.82	-2.21	-0.552	0.609	2.371	0.168	83.25
	X7	74.18	75.25	-1.07	-0.268	0.144	0.559	0.479	52.1

Table 5. Estimating significance of study using simple ANOVA analysis

Sum of Square (Total)	Sum of Square (Within)	Sum of Square (Between)	Degree of Freedom		F-value	P-value	Confidence level (%)
			Numerator	Denominator			
713.393	445.940	267.455	3	28	5.597	0.004	99.61

for drying in a hot air oven at 45^{0}C (Sathendra et al. 2019; Zhu et al. 1995). The dried rice straw was ground and sieved further to produce powdered raw material of size 30-50 mm. The powdered rice straw substrate was pretreated using the dilute sodium hydroxide and autoclaving after that10 gram of substrate was mixed with 80 ml of 0.5 M NaOH solution (Khanahmadi et al. 2018; Zhu et al. 2006). The mixture was autoclaved 121^{0}C at 15 psi for 10 minutes. After cooling the residue was washed thoroughly with distilled water until neutral pH is reached and oven dried at 65^{0}C and weighed for further enzymatic hydrolysis. Screening of medium component affecting the production of xylose and glucose using Plackett–Burman design: Screening of significant medium component affecting at the utmost level the microbial enzymatic hydrolysis of pretreated rice straw was performed using Plackett–Burman design (Thi et al. 2018; Plackett and Burman 1946). The following Identified independent variables including C-source, N-source, and some inorganic ions, were selected for analysis viz. Malt extract (X1), Ammonium sulphate (X2), KH_2PO_4 (X3), $MgSO_4$. $7H_2O$ (X4), Glucose (X5), $FeSO_4$. $7H_2O$ (X6) and Peptone (X7) (Mandels et al. 1974; Aggarwal et al. 2017). Each independent variable was examined at two levels, a high (+1) and a low (-1) level indicating concentration range of each parameter (Table 1). The screening experiments were conducted as per Plackett–Burman design matrix (Table 2). Inoculum at a rate of 3 ml each of Trichodermareeseiand Aspergillustamarii was transferred into separate Erlenmeyer flask of 250 ml capacity each containing the 50 ml of microbial growth medium along with 5 gram of alkali pretreated rice straw. The pH of the medium was adjusted to 6.5. The sample containing

Erlenmeyer flasks were incubated at 28⁰C in a rotary shaker for 7 days at 160 rpm solution (Zhu et al. 2015). All experiments were carried out in the triplicate run.

Estimation of xylose and glucose: To express the accuracy, 1 ml of solution was collected from each flask at 24 hr interval for 4 days and was subjected to centrifugation at 8000 rpm at 4⁰C in a cooling centrifuge for 10 min, the supernatant obtained was further analyzed for xylose and glucose concentrations. The amount of xylose content in the hydrolysate was estimated by phloroglucinol method (Miller 1959 Jamaldheen et al. 2019). The amount of glucose content in the hydrolysate was determined by DNS method (Eberts et al. 1979 Sorn et al. 2019). Estimation of xylose: The solution of phloroglucinol ($C_6H_6O_3$), prepared by dissolving 0.5 gram of $C_6H_6O_3$ in100 ml of glacial acetic acid (CH_3COOH) and 10 ml of concentrated HCl. This forms a coloured reagent. 200 µl of supernatant was mixed with 5 ml of colour reagent and boiled for 5 min in boiling water bath. After cooling it, the absorbance was recorded in UV spectrophotometer at 554 nm, and the concentration of xylose was estimated against a standard xylose curve prepared (Miller 1959 Jamaldheen et al. 2019).

Estimation of Glucose: About 0.5 ml of sample was drawn from every treatment into test tubes, and the volume was made up to 3ml using distilled water. 3 ml of 3,5-dintrosalycylic acid (DNSA) reagent was added to each sample, mixed well after which the sample tubes were heated in a water bath for 5 minutes and then cooled thereafter. After cooling the absorbance was recorded in UV spectrophotometer at 540 nm and the concentration of glucose was estimated against a standard glucose curve prepared (Eberts et al. 1979; Sorn et al. 2019).

RESULTS AND DISCUSSION

Screening of medium components to enhanced fermentable sugars by Plackett–Burman design: Plackett-Burman design was used to efficiently select and screen critical nutritional variables of the growth medium of A. tamarii, and T. reesei contributed to enhanced fermentable sugars yield response from alkali pretreated rice straw by enzymatic hydrolysis under shake flask fermentation. The filamentous fungi such as Aspergillus sp. and Trichoderma sp. have been reported to produce cellulases and xylanases enzymes in a single fermentation system (Cekmecelioglu and Demirci 2018; Jampala et al. 2017). Each independent medium component has been assigned at two concentration level and as low (-1) and high (+1) coded values in the Plackett-Burman design (Table 1). The composition of the medium used for microbial growth over alkali pretreated rice straw and individual medium component affecting the enhanced release of fermentable sugar by enzymatic hydrolysis were screened with 7 medium component in 8 experimental runs in which the productivity of xylose and glucose was recorded as yield response as shown in Table 2. All the experiments were carried out as per the Plackett-Burman design matrix, and each run was conducted in triplicate and mean value was recorded against each response (Table 2). Plackett-Burman design method has been reported as an effective statistical tool in screening various factor responsible for enhanced release of xylose and glucose from lignocellulosic biomass (Singhania et al. 2007).

Effect of medium component on the yield of xylose and glucose in A. tamarii broth: Screening of nutrient medium component influencing the production of fermentable sugar especially pentose (xylose) and hexose (glucose) was analyzed and screened by Plackett-Burman experimental design. The result is presented in Table 3. Among seven nutrient components used in study, the Monopotassium Phosphate and Glucose were tested at 95.95% and 99.33% significance level respectively with p-value <0.05 found a significant factor influencing the maximum xylose concentration release of 36.03 mg/g in the A.tamarii broth in run 7 (Table 2). Monopotassium Phosphate plays an important role in A. tamarii metabolism affecting significantly enhanced release of xylose sugar in fermentation broth (Zhao et al. 2018; Maciel et al. 2008). The glucose screened as important carbon source in medium tested at a significance level of 99.33% with p-value <0.05 contributed

significantly towards the enhanced release of xylose in broth. Previous studies reported the effect of the synergistic action of glucose as a carbon source for enhanced release of xylose in A. tamarii broth when supplemented with alkali pretreated rice straw (Karunakaran et al. 2014). All other medium components were screened out as the less significant medium component for xylose release in broth found with p-value >/= 0.05 and significance level <95%. The medium component ammonium sulphate was tested at confidence level of 99.7% with p value<0.05 (Table 3) identified as an important source of inorganic nitrogen in the medium towards enhanced release of glucose in broth with a concentration of 29.84 mg/g in run 7 (Table 2) by cellulolytic enzymatic action of A. tamarii shows good agreement with previous findings (Lee 2018; Gautam et al. 2011). **Effect of medium component on the yield of xylose and glucose in T.reesei broth:** Enhanced yield of xylose in the *T.reesei* broth attributed to magnesium sulphate as a significant medium component with a significance level of 100% at p value<0.05 (Table 4) indicating it as an important enzymatic cofactor (Fortkamp and Knob 2014) in releasing maximum xylose concentration of 22.15 mg/g in broth in run 5 (Table 2).

Glucose identified as a significant medium component with significance level of 99.88% at p value<0.05 for maximum xylose release by enzymatic action of T.reesei may be due to the presence of pentose sugar arabinose because of rice straw pretreatment and hydrolysis which might have enhanced enzymatic activity of *T.reesei* leading to maximum xylose release in the broth (Sorn et al. 2019; Xiong et al. 2004). Peptone with significance level tested at 97.37% at p-value <0.05 in the current study has also been identified as the best nitrogen source for the improved enzymatic action of T.reesei leading to the enhanced release of xylose in broth. Similar results were also reported previously for nitrogen source for the improved enzymatic action of T.reesei leading to the enhanced release of xylose (Gupta et al. 2018). Glucose sugar concentration was recorded as highest with a value of 24.31 mg/g in run 5 by the enzymatic action of T.reesei (Table 2) with significant medium component screened and identified as malt extract, ammonium sulphate and glucose (Table 4). Each component tested at a significance level of 97.37%, 96.71 and 99.99% respectively at p-value <0.05. The other medium component were screened out as the less significant medium component with p-value >0.05. Secretion of active cellobiohydrolase I and the endoglucanase I catalytic core domain into the culture medium were induced greatly when the Trichoderma reesei was grown on glucose-containing medium (Cekmecelioglu and Demirci 2018; Nakari and Penttilä 1995) . Enhanced released of glucose in the broth by the enzymatic action of T.reesei may also be due to an additional 15-20% carbon source of malt extract along with glucose in the medium as reported previousaly (Bagewadi et al. 2017 and Jamaldheen et al. 2019).

Ammonium sulphate has been reported as best nitrogen source in an earlier study (Guoweia et al. 2011) towards the enhanced enzymatic activity of T.reesei which finds good agreement in the current study with this factor tested at a confidence level of 99.92% (Table 4) towards the enhanced release of glucose in the broth by microbial enzymatic action.All the factors were tested at α value of 0.05 and 95 % confidence level. The significance of overall study was estimated using simple ANOVA test at the confidence level of 95%. The p-value at 95% confidence level was found to be 0.004 with F-value of 5.59 validating the experimental trail as significant (Table 5).

CONCLUSION

The medium component used for growth of T.reesei and A.tamarii were statistically screened comparatively as per the Plackett-Burman design and effect of each independent medium component on the yield of glucose and xylose concentration in the final broth were estimated. Finally, it is concluded that the microbial strain A.tamarii was found better than T.reesei for enzymatic hydrolysis of alkali pretreated rice straw leading to the enhanced release of fermentable sugar viz., xylose and glucose.

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Effects of *Ocimum sanctum* and *Allium sativum* Extracts Against Diabetes and Determination of DNA Damage and Cytotoxicity in Alloxan Induced Diabetic Rats

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ABSTRACT
Diabetes is associated with imbalance of antioxidant defense mechanism, causes alteration in various biomolecules, including DNA and increases the level of serum glucose, superoxide anion, and hydrogen peroxide. It is known to induce toxic effect on pancreas, liver, brain and biochemical parameters.The excessive reactive oxygen species (ROS) damage lipid and DNA. Reactive oxygen species are responsible for histological changes in architecture of liver, brain tissue. Alloxan induced diabetes produces oxidative stress induced damage in rat tissues. Diabetic rats treated with oral administration of aqueous suspension of *Ocimum sanctum* and *Allium sativum* daily for 14 days showed decrease in the level of glucose, superoxide anion, hydrogen peroxide, tail lengh of DNA damage and regeneration of liver, brain tissues cells. Results showed that diabetic group treated with aqueous suspension of *Ocimum sanctum* and *Allium sativum* reduced the level of oxidative stress, cytotoxicity and also serum blood glucose levels.

KEY WORDS: DIABETES, DNA DAMAGE, HISTOPATHOLOGY, OCIMUM SANCTUM, ALLIUM SATIVUM.

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disease. It is one of the most challenging health problems in the 21stcentury (Narayan et al., 2017). The prevalence of diabetes is increasing globally and the number of diabetics is expected to increase to 366 million by 2030 (Zimmet et al., 2017). Diabetes is associated with increased oxidative stress that results in damage of several cellular biomolecules such lipid, carbohydrates and protein (Tan et al., 2018). Oxygen-free radicals induce a variety of lesions in DNA, including oxidized bases, abasic sites, DNA strand breaks and formation of cross-links between DNA and proteins (Klages-Mundt et al., 2017). Bigagli et al., (2019) reported that the products generated by oxidative DNA damage are significantly elevated in diabetes mellitus (DM) and the pattern of modification was the same as one expected from the attack of the hydroxyl radical (OH•) upon DNA. Moreover, it has been shown that hydroxyl radical is produced by the Fenton reaction in the presence of transition metal ions is responsible for DNA damage (Liguori et al., 2018).

Oxidative stress is the imbalance between the production and scavenging of ROS and biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage (Andersson et al., 2018). Disturbances in the normal redoxstatus of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (Roy et al., 2017). In humans, oxidative stress is involved in many diseases including atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, schizophrenia, bipolar disorder, fragile X syndrome, etc.(Manna et al., 2018). Several drugs, xenobiotics and environmental pollutants are known to cause this imbalance between formation and removal of free radicals (Aguilar et al., 2016). Biological antioxidants including vitamins can prevent this uncontrolled formation of free radicals and activated oxygen species or inhibit their reaction with biological structures (Tan et al., 2018). The destruction of most free radicals and ROS rely on the oxidation of endogenous antioxidants and reducing molecules (Ighodaro et al., 2018). It is observed that in case of Alloxan induced diabetes, DNA damage comet tail length increases (Zhong et al., 2018). The present study was aimed to evaluate the possible cytoprotective effect of the *Ocimum sanctum* and *Allium sativum* plant extracts and determine the antidiabetic propertes of O. sanctum and A. sativum plant extracts in the treatment of type I diabetic rats, compared to normal control and untreated diabetic rats.

MATERIAL AND METHODS

Experimental animals: Male albino rats of Wistar strain (weight 120 ± 20 g) were used in the proposed study. Animals were obtained from the animal house facility of the Defense Research and Development Establishment, Gwalior, India, and were maintained under controlled conditions of temperature (25 ± 20C), relative humidity of (50 ± 15%), and normal photoperiod (light-dark cycle of 12 h). The rats were maintained in the animal room of School of Studies in Biochemistry on standard pellet diet and tap water ad lib. Animals were housed throughout the experiment in polypropylene cages containing paddy husk as bedding and allowed to acclimatize to the environment of animal room for 7 days before the start of experiment.

Experimental design: Thirty six rats were randomly divided into six groups of six rats each. Animals were divided into six groups and were given following treatments:

Group 1: Control (normal blood glucose level).

Group 2: Treated control group (treated with aqueous suspension of O. sanctum 2.5 mg/kg body weight).

Group 3: Treated control group (treated with aqueous suspension of A. sativum 0.25 mg/kg body weight).

Group 4: Diabetic (I.V. injection of alloxan 70 mg/kg body weight).

Group 5: Treated diabetic group (treated with aqueous suspension of O. sanctum 2.5 mg/kg body weight).

Group 6: Treated diabetic group (treated with aqueous suspension of A. sativum 0.25 mg/kg body weight).

Induction of experimental diabetes and plant extract treatment: Type I diabetes was induced by giving single intravenous injection of alloxan monohydrate 70 mg/kg body weight, dissolved in 0.9% solution of sodium chloride (Misra et al., 2012). The animals were checked for blood glucose level after 48 h and rats with blood sugar level above 200 mg/dl were used for the experiment.*Ocimum sanctum* (Tulsi) leaves were sourced from the Botanical Garden of Jiwaji University, Gwalior, and were cleaned and aqueous extract was prepared. A total of 2.5 mg/kg body weight of this extract was given orally to the rats of group 2 and 5 with the help of cannula, daily for two weeks. *Allium sativum* (Garlic) seeds were purchased from the local herbal market, cleaned, and aqueous extract was prepared and administered art the rate of 0.25 mg/kg body weight orally to the rats of group 3 and 6 with the help of cannula, daily for two weeks. Rats were humanely killed after the last treatment by cervical dislocation. The different tissues were excised off, washed with 0.9% NaCl and used for different estimations. Animals were handledand treated ethically and were sacrificed humanely as per rules and instructions of Ethical Committee of Animal Care of Jiwaji University, Gwalior, India, in accordance with the Indian National law on animal care and use.

Superoxide anion: Superoxide anion release was measured by superoxide dismutase inhabitable reduction of ferricytochrome-c (Cohen et al., 1980). The tissue homogenate was incubated in PBS-EDTA buffer (EDTA 5 mM, pH 7.4) with phorbol-12, 13-myristate (PMA) at 370C for 15 min. The total assay volume was 1 ml, the final concentrations of ferricytochrome-c and PMA were 50 μmol/L, and 100 nmol/L, respectively. The change in absorbance was measured spectrophotometrically at 550 nm for 10 min with a double beam Shimadzu UV-1800A spectrophotometer at room temperature. The amount of superoxide anion secreted into the medium was calculated on the basis of the molar extinction coefficient of reduced cytochrome c as 2.1×104 M−1 cm−1 and values are expressed as n mole $O_2^{.-}$/ min. Hydrogen peroxide: Hydrogen peroxide in tissue was measured by the method of Pick et al., (1986). For assay of H_2O_2, 100 μl of tissue homogenate prepared in Tris-HCl buffer (0.02M, pH 7.5), 100 μl of assay solution (containing 0.2 ml phenol red, 0.2 g/l and 0.2 ml of horse radish peroxidase, 20 U/ml in potassium phosphate buffer, 0.05 M, pH 7.0 and 9.6 ml of 0.9% NaCl), was taken and reaction was started by the addition of 10 μl of 1.0 N NaOH. Absorbance was recorded at 600 nm in a microplate using ELISA reader. Hydrogen peroxide standard curve

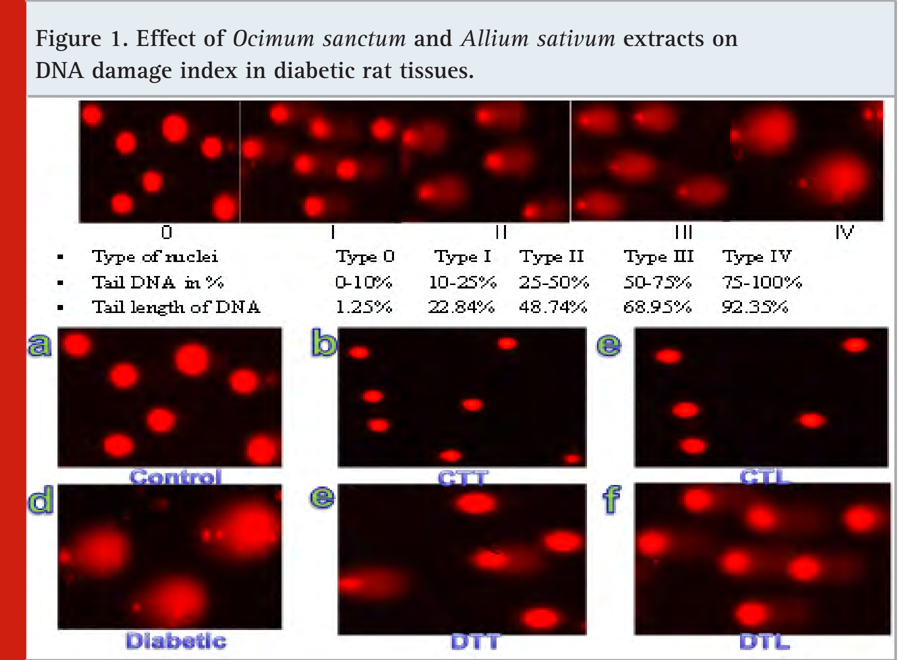

Figure 1. Effect of *Ocimum sanctum* and *Allium sativum* extracts on DNA damage index in diabetic rat tissues.

was plotted by taking different concentrations of H_2O_2, ranging from 20 to 100 µmol in a total volume of 100 µl and processed in the same way. Results are expressed as µmol H_2O_2 formed ml-1 preparation. Single cell gel electrophoresis (SCGE) or Comet assay: Single strand breaks were measured by alkaline comet assay as described by Sasaki et al., (1997). Fresh tissues were collected and homogenates (25% w/v) were prepared in homogenizing buffer (0.075 M NaCl containing 0.024 M EDTA, pH 7.5) with a single stroke.To obtain nuclei, homogenates were centrifuged at 700 g for 10 min and the resulting pellets were gently resuspended in 4.0 ml of chilled homogenizing buffer.Seventy five µl of normal melting agarose (1% prepared in 0.1 M sodium phosphate buffer, pH 7.2 containing 0.9% NaCl) was quickly layered on an end frosted slide, covered gently with another slide, and allowed to solidify.The upper slide was gently removed and the precoated slide was coated with 100 µl of mixture containing equal volumes of sample (nuclei preparation) and low melting agarose (2% in phosphate buffer saline). Slides were then immersed in the lysis buffer (containing 0.25 M NaCl, 100 mM EDTA, 10 mMTrizma base, 1% sarcosine, pH 10.0 adjusted with 10 N NaOH; 5% DMSO and 1% Triton X-100 was added just before use) for 1 h at 4°C in the dark. After lysis, the slides were rinsed with chilled distilled water, transferred on a horizontal electrophoresis platform and immersed in electrophoresis buffer (300 mM sodium hydroxide and 1 mM EDTA, pH > 13.0) for 20 min for unwinding of DNA. Electrophoresis was performed for 20 min at constant voltage (1 V/cm and 300 mA). After electrophoresis, the slides were washed thrice with neutralizing buffer (0.4 M Tris-HCl, pH 7.4) for 5 min each.Slides were dehydrated in absolute methanol for 10 min and left at room temperature to dry. The whole procedure was performed in dim light to minimize artefactual DNA damage. Just before visualization, each slide was stained with 50 µl of ethidium bromide (20 µg/ ml), rinsed with water, and covered with a cover slip. A total of 50 cells were scored per tissue per animal (25 from each replicate slide). Analyses were performed on the basis of the type of comet visualized on the slide. The nuclei were counted and divided into five types as stage 0, 1, 2, 3 and 4.The DNA damage index was calculated as # 0 + # 1 + # 2 + # 3 + # 4/ # of cell scored. Two independent experiments were conducted in each treatment. The animal (and not the cell) was used as the experimental unit. Slides were viewed under fluorescence microscope (Leica 4000B Digital Microscope). Analyses were performed at 100X magnification, with a Leica Optiphase microscope equipped with an excitation filter of 515-560 nm and barrier filter of 590 nm. Histopathology: For histopathological analyses, tissues were collected at the time of sacrifice, freed from fat bodies,

Figure 2. Histology of liver tissue of control (a), control treatment (b, c), alloxan induced diabetic (d) and *Ocimum sanctum* and *Allium sativum* extract treated diabetic (e, f) rats.

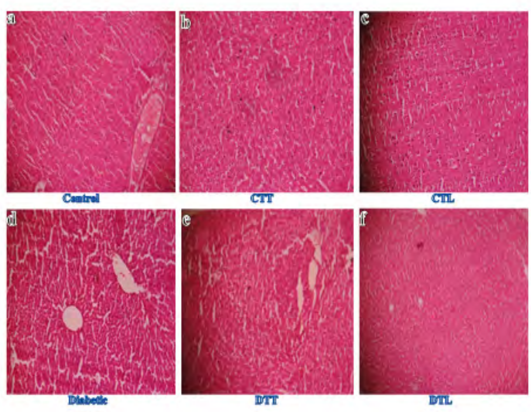

Figure 3. Histology of brain tissue of control (a), control treatment (b, c), alloxan induced diabetic (d) and *Ocimum sanctum* and *Allium sativum* extract treated diabetic (e, f) rats.

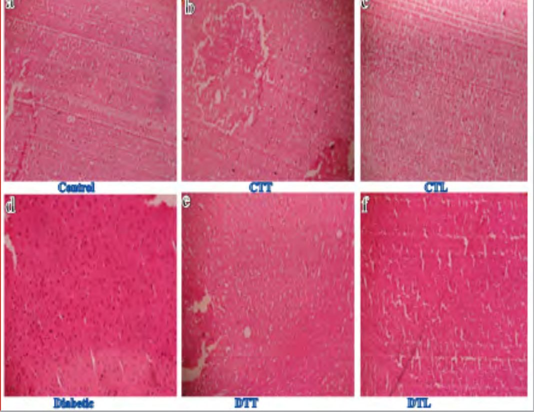

Table 1. Effect of oral treatment of extracts of *Ocimum sanctum* and *Allium sativum* for 7, 14 days on levels of level of blood glucose in control and alloxan induced diabetic rats.

S No.	Groups	Level of glucose in experimental animals		
		0 day	7th day	14th day
1.	Control	103.67±2.4	109.00±2.65	111.67±3.53
2.	Control + *Ocimum sanctum*	109.67±1.76#	106.33±1.86#	100.67±2.6#
3.	Control + *Allium sativum*	110.33±2.6#	107.67±1.76#	102.67±2.96#
4.	Diabetic	413.00±8.14***	419.00±6.66***	422.33±6.36***
5.	Diabetic + *Ocimum sanctum*	421.00±7.21***	320.67±3.48***	237.67±6.17***
6.	Diabetic + *Allium sativum*	418.67±4.63***	326.67±3.28**	248.33±2.91***

Glucose concentration is expressed as mg/dl. Values are given as mean ± S.E. of six set of observation. Values are significance at $p>0.05$ #, $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. Diabetic rats were compared with control rats, Ocimum sanctum and Allium sativum treated diabetic rats as compared with diabetic control rats.

Table 2. Effect of oral treatment of extracts of *Ocimum sanctum* and *Allium sativum* for 14 days on levels of superoxide anion in the tissues of control and alloxan induced diabetic rats.

S No.	Groups	Brain	Liver
1.	Control	4.76±0.28	9.68±0.42
2.	Control + *Ocimum sanctum*	3.97±0.16#	8.10±0.27*
3.	Control + *Allium sativum*	4.36±0.21#	8.89±0.42*
4.	Diabetic	11.98±0.35***	20.00± 0.55***
5.	Diabetic + *Ocimum sanctum*	7.05±0.20***	12.70± 0.16***
6.	Diabetic + Allium sativum	8.02±0.21***	13.33± 0.27***

Superoxide anion concentration is expressed as n mole. Results are expressed as mean ± S.E. of six set of observation. Significance is based on $p>0.05$#, $p<0.05$*, $p<0.01$**, $p<0.001$ Diabetic rats were compared with control rats, Ocimum sanctum and Allium sativum treated diabetic rats as compared with diabetic control rats.

Table 3. Effect of oral treatment of extracts of *Ocimum sanctum* and *Allium sativum* for 14 days on the levels of hydrogen peroxide in the tissues of control and alloxan induced diabetic rats.

S No.	Groups	Brain	Liver
1.	Control	52.42±0.40	50.45±0.26
2.	Control + *Ocimum sanctum*	47.88±0.40*	46.54±1.68#
3.	Control + *Allium sativum*	49.84±0.15*	48.34±0.15*
4.	Diabetic	120.00±0.26***	92.88±1.58***
5.	Diabetic + *Ocimum sanctum*	79.55±1.05***	60.15±0.84***
6.	Diabetic + *Allium sativum*	84.24±1.18***	64.99±0.79***

Hydrogen peroxide concentration is expressed as μ mole/ml. Results are expressed as mean ± S.E. of six set of observation. Significance is based on $p>0.05$#, $p<0.05$*, $p<0.01$**, $p<0.001$*** Diabetic rats were compared with control rats, Ocimum sanctum and Allium sativum treated diabetic rats as compared with diabetic control rats.

washed with normal saline and fixed in Bouin's fluid for 12–24 h. After fixation, tissues were washed overnight under running tap water to remove excess fixative, and embedded in paraffin blocks. Paraffin blocks were cut at 4 µm for liver and pancreas and at 12 µm for brain with the help of semi-automated microtome (Leica EG 1106 Microtome). Six slides per tissue were prepared and stained with hematoxylin and eosin (McManus et al., 1960). Stained tissue sections were mounted in DPX, covered with cover slip and viewed under light microscope at 10X magnification (Leica Optiphase microscope).

Statistical analysis: Results are expressed as mean ± S.E. of different sets of observation taken on different days.All the statistical analyses were performed using one-way analysis of variance (ANOVA) with post hocDunnett's multiple comparison test applied across treatment groups for each tissue.Significance level was based on $p< 0.05$.

RESULTS

Blood glucose: The blood glucose level of all the rats was tested by taking the blood from the tail vein and using electronic glucometer. Administration of alloxan (70 mg/kg, i.v) led to 4-fold elevation of fasting blood glucose levels, which was maintained for a period of 2 weeks (Table 1).It was observed that oral administration of aqueous extract of *Ocimum sanctum* and *Allium sativum*, significantly decreased the blood glucose levels in diabetic as compared to the blood glucose level of control rats. The results of the present study showed that oral administration ofextract of O. sanctum and *Allium sativum* daily for 14 days, to the diabetic rats caused 23.5%, and 22% decrease on 7th day and 43.72%, and 41.2% decrease inthe blood glucose level on day 14th of the start of treatmet when compared with respective untreated diabetic rats.The results clearly showed the hypoglycemic potential of O. sanctum and A. sativum extracts.

Superoxide anion: Superoxide anion (O2.–) production in diabetic rat tissues was monitored by the superoxide dismutase inhabitable cytochrome-c reduction assay. Diabetic rats showed 106.6% and 151.7% increase in O2.–production in the liver and the brain when compared with control (Table 2). Diabetic rats given oral administration of O. sanctum and A. sativum extracts daily for 14 days, showed36.5%, and 33.4% decrease in the rate of production of O2.– in the liver and 41.2%, 33.1% in the brain superoxide anion production when compared with diabetic rats. Feeding of extracts of O. sanctum and A. satiuam to the control rats 14 days,showed 16.3%, and 8.1% decrease in the rate of production of O2.– radicals in the liver while 16.6% and 8.4% decrease in superoxide anion production was observed in the brain when compared tissues of untreated control rats.

Hydrogen peroxide: Levels of hydrogen peroxide also increased in the tissues of diabetic rats. The results showed that there was significantly high concentration of H2O2 in the liver and the brain of diabetic rat tissues. An increase of 128.9% and 84.1% in the level of H2O2 in the liver and the brain of diabetic rats was observed when compared with control. Oral administration of extract of O. sanctum and A. sativum for 14 days to diabetic and control rats showed reduction in the level of H2O2.The treated diabetic rats showed 33.7% and 29.8% decrease in the liver and 35.2% and 30% decrease in the brain levels of H2O2 as compared with diabetic rat tissues, respectively. Control rat tissues treated with O. sanctum and A. sativum extracts for 14 days, showed 8.2%, 4.9% decrease in the liver and 7.8%, 4.2% decrease in the brain H_2O_2 levels when compared with unteated control rat tissues, respectively. The same O. sanctum and A. sativum treatment of diabetes caused 51.8%, 60.7% increase in the liver and 19.2%, 28.8% increase in the brain H2O2 levels when compared with control rat tisuues (Table 3).

Diabetes induced DNA damage: Results of the present study clearly showed that intravenous injection of alloxan caused significantly marked DNA damage in all the rat tissues examined, as evidenced by the increase in the number of nuclei of Type I, II, III and IV. In the tissues of control rats, almost all the nuclei were of Type 0, the typical condensed type, round nuclei indicating intact DNA. The result showed that a single I. V.

dose of alloxan caused 1304.5% and 1471.4% increase in DNA damage index in liver and brain tissue of rats, when compared with control rats' tissue and measured diabetic rats' tissue after 14 days of alloxan exposure (Table 4). Results showed that oral administration of O. sanctum and A. sativum plants extract daily for 14 days, to the diabetic rats caused 74.8%, 68.6% decrease in DNA damage index in liver and 75.2%, 69.1% decrease in DNA damage index in brain when compared with diabetic untreated group. O. sanctum and A. sativum treatmemt for 14 days to the control rats showed 13.6%, 9.1% decrease DNA damage index in liver and 33.3%, 9.5% decrease in DNA damage index in brain when compared with control group tissue (Figure 1).

Histopathological studies in diabetes

The light microscopic changes in the tissues of alloxan exposed animals were compared with respective controls. The liver of control and control treated oral O. sanctum and A. sativum plants extract for two weeks rats showed normal histopathological structure of the central vein and surrounding hepatocytes (Figure2a, 2c and 2d). Alloxan exposed rats showed severe dilation and congestion of the central vein with degeneration in the surrounding hepatocytes in the liver.Inflammatory cell infiltration in portal area, surrounding the congested portal vein with degeneration in the hepatocytes, was observed in alloxan treated rats liver. Diffuse kupffer cells proliferation between the degenerated hepatocytes was also observed in some treated tissues.The hepatic blood vessels showed congestion, fibrosis and infiltration of red blood cells (Figure2b) When the diabetic rats were given O. sanctum and A. sativum oral treatment for two weeks and its showed regenerating liver tissue (Figure2e and 2f) which could be comparable to that of non-diabetic control rats (Figure2).

The light microscopic changes in brain of experimental animals were compared to those of controls. Alloxan exposure caused degenerative morphological changes in brain and these changes were clear in brain of rats receiving single dose of alloxan. Mild ischemia was seen in brain of rats and Changes in nuclear shape and chromatin condensation were seen in the brain of rats. Alloxan exposure caused marked changes in brain including affected granular cell neurons in cerebrum, disruption of cerebral cortex with neuronal loss and gliosis (Figure3b). These diabetic rats were given O. sanctum and A. sativum oral treatment for two weeks and its showed regenerating brain tissue (Figure3e and 3f) which could be comparable to that of non-diabetic control rats. These histological observations showed the protective role of polyphenolic extract on brain in alloxan induced diabetic rats. Result of brain control and control treatments O. sanctum and A. sativum tissue rats showed normal nuclear shape, chromatin condensation of brain rats (Figure3a, 3c and 3d) and no histopathological alterations were observed in these animals (Figure3).

DISCUSSION

ROS play an important role in the deterioration of diabetes, causing high chemical reactivity and capable of damaging lipids, proteins and DNA (Nita et al., 2016). Further, it can be stated that the free radical scavenging property reduces the DNA damage in alloxan induced diabetic rats. Higher levels of MDA and 4-HNE, resulted from lipid peroxidation process were reported in the alloxan induced rats, which could be due to the poor glycemic control and high production of free radicals. MDA and 4-HNE are known to interact with DNA (Liguori et al., 2018). Such interactions can cause DNA damage, which can lead to cytotoxicity and genotoxicity (Lui et al., 2016). Recently, significant increase in the level of 8-oxodeoxyguanosine, a marker for oxidative damage have been reported in the diabetic rat tissues (Ortiz et al., 2016).

As cellular enzymes efficiently repair DNA damage, its measurement gives a snapshot view of the level of oxidative stress, in contrast to measurement of oxidation of other biomolecules which are not repaired and have a slow turnover such as lipids or proteins. DNA oxidation may therefore be of considerable value in following the progress of the disease and its metabolism (Nasif et al., 2016). Comet assay was performed to detect the oxidative DNA damage and alkylated bases in diabetes. Oxidative stress depicts the

existence of products called free radicals and ROS which are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems (Fang et al., 2002). There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress (Johansen et al., 2005, Rosen et al., 2001). Free radicals are formed disproportionately in diabetes by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins (Obrosova et al., 2002). Abnormally high levels of free radicals and simultaneous decline of antioxidant defence systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of diabetes mellitus (Maritim et al., 2003).

It is well established that oxidative stress is produced under diabetic conditions through multiple sources causing an increase of hydroxyl radicals (Turko et al., 2001). A hydroxyl radical in turn produces a multiplicity of modifications in DNA. Oxidative attack by OH radical on the deoxyribose moiety of DNA is lead to the release of free bases from DNA, generating strand breaks with various sugar modifications, nucleotide modifications, particularly in sequences with high guanosine content (Hegde et al., 2008) and simple a basic (AP) sites. In fact, one of the major types of damage generated by Reactive Oxygen Species (ROS) is AP site, a site where a DNA base is lost. The oxidative DNA damage occurs in their peripheral blood lymphocytes (Sardas et al., 2001) and the DNA damage in tissue, lymphocytes and leucocytes can be used as a marker of oxidative stress in diabetes (Pitozzi et al, 2003). Additionally, it has been demonstrated that DNA damage was significantly higher in the poorly controlled diabetic patients compared to well control subject, regardless of sex (Dinçer et al., 2002). Van Loon et al., (1992) showed significantly increased basal levels of DNA damage in whole blood. ROS induces several types of lesions in DNA, including single or double-strand breaks, alkali-labile sites, and various species of oxidized purines and pyrimidines, which are easily detected by alkaline comet assay (Nikitaki et al., 2015).

Table 2. **Effect of oral treatment of *Ocimum sanctum* and *Allium sativum* for 14 days on DNA damage index in the tissues of alloxan induced diabetic rats.**

S No.	Groups	Brain	Liver
1.	Control	0.21±0.02	0.22±0.02
2.	Control + *Ocimum sanctum*	0.14±0.01*	0.19±0.01*
3.	Control + *Allium sativum*	0.19±0.01#	0.20±0.01*
4.	Diabetic	3.30±0.15***	3.09±0.03***
5.	Diabetic + *Ocimum sanctum*	0.82±0.01***	0.78±0.01***
6.	Diabetic + *Allium sativum*	1.02±0.01***	0.97±0.02***

DNA damage is expressed as damage index level of DNA. Values are given as mean ± S.E. of six set of observation. Values are significance at p>0.05 #, p<0.05 *, p<0.01 **, p<0.001 ***. Diabetic rats were compared with control rats, Ocimum sanctum and Allium sativum treated diabetic rats as compared with diabetic control rats.

A large variety of oxidized bases have been identified in nuclear DNA but 8-oxo-7,8-dihydroguanosine (8-oxoGua) is one of the most abundant and readily formed. The 8-oxoGua in DNA mispair with adenine during replication. Thus presence of 8-oxoGua in DNA may lead to transversion mutations. It has been suggested that this kind of lesion play an important role in the initiation, promotion and progression of tumors. The high levels of oxidized bases in patients infected with the human immunodeficiency virus (HIV) are reported which might influence the progression of the infection into acquired immunodeficiency syndrome (Olinski et al., 2002). High levels of 8-oxo-Gua have also been found in lesions of the aorta wall in atherosclerosis patients (Olinski et al., 2002). DNA oxidative damage has also been linked to other diseases, notably Alzheimer's disease, Huntington's disease and Parkinson's disease (Cooke et al., 2005).

CONCLUSION

Alloxan induced diabetes mellitus is associated

with elevated level of oxidative DNA damage, increase in the level of blood glucose, superoxide anion, hydrogen peroxidesusceptibility to cytotoxicity and the decrease efficacy of DNA repair. Diabetic rats treated with oral administration of O. sanctum and A. sativum aqueous extracts decrease in the level of blood sugar, superoxide anion, hydrogen peroxide, length of DNA tail, decrease cytotoxicity and increase efficacy of DNA repair. Further investigation of the mechanism of action of this herbal plant leave extracts against diabetes.

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Genetical
Communication

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Genetic Analysis of Ticks from Livestock of Akola District Maharashtra India

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ABSTRACT

DNA from seven isolates of ixodid ticks was collected from the livestock in and around Akola District Maharashtra India, and was analyzed by RAPD using PCR. Selected three casual primers were used for the study of genetic analysis among different isolates of ixodid ticks. A high degree of genetic polymorphism with a unlike pattern of RAPD profiles for each tick isolate was identified with all these random primers. This variability was also established by similarity coefficient values and dendrogram which were perform using mean RAPD profiles for all the primers between different isolates of ticks. The conclusion suggest existence of a complex genotypic diversity of the ixodid tick in Akola district, first time.

KEY WORDS: TICKS, RANDOM PRIMERS, RAPD, GENETIC VARIABILITY of IXODID TICK, AKOLA.

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INTRODUCTION

India is house for approximately 109 tick species, under 12 genera (Ghosh et al., 2007)... The larva, nymph and adult stages are all parasitic as they suck on blood and lymph of their hosts and thus are the vectors of many kinds of pathogens. A basic awareness on genetic diversity of tick population help in understanding the susceptibility or resistance nature of ticks for effective control as well as production of vaccines (Passos et al., 1999). The molecular techniques or tools that are dramatically advanced the knowledge on the control of ticks and the diagnosis of tick-born diseases (Stiller, 1992). Studies on genomic variations within a species have great impact on understanding the pathogen transmission, the epidemiology of the illness and its control.

Random Amplified Polymorphic DNA (RAPD) fingerprinting allows detecting genetic polymorphism using arbitrary primers (Welsh and McCelland, 1990; Williams et al., 1990). Lan et al., (1996) which offers a rapid and efficient method for generating a new series of DNA markers in animal species, (Mishra et al., 2012, Amany et al., 2019).) The results obtained out of such study shows that the primers are diagnostic

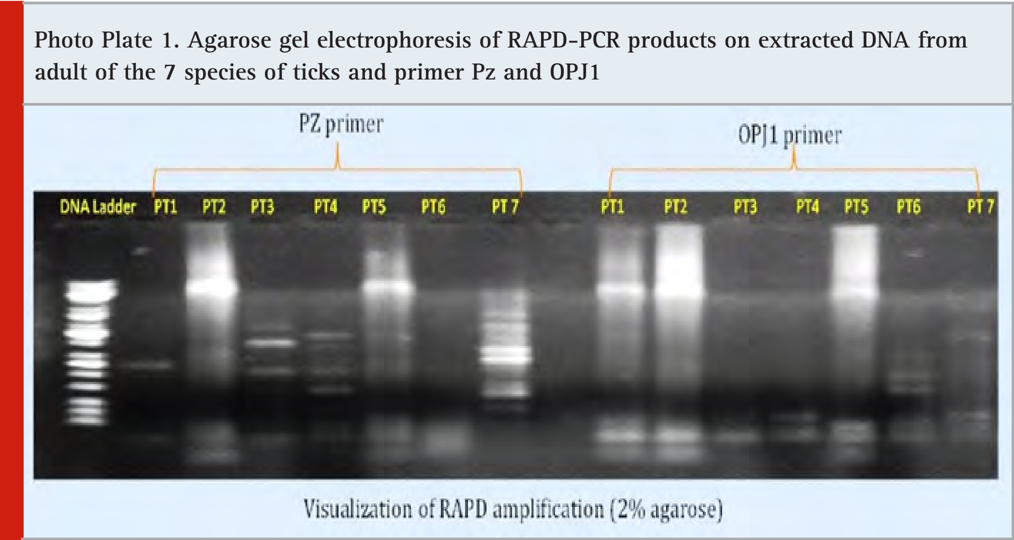
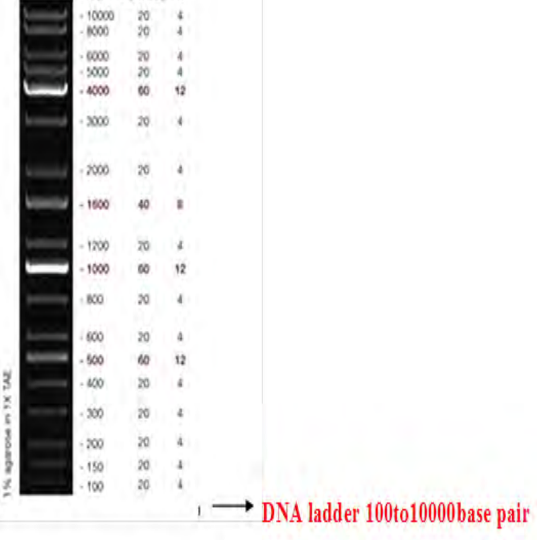


Photo Plate 1. Agarose gel electrophoresis of RAPD-PCR products on extracted DNA from adult of the 7 species of ticks and primer Pz and OPJ1

PT1: *Rh. Boophilusmicroplus*
PT2: *Hyalomma marginatum isaaci*
PT3: *Hyalomma a. anatolicum*
PT4: *Hyalommahussaini*
PT5: *Rh. Annulatus*
PT6: *Rh. Haemaphysaloides*
PT7: *Haemaphysalisbispinos*



and variations in different related species under study (Abdul Anvesh et al., 2018). Morphological misidentification could be reduced significantly after using Multiplex-PCR as the pairs of closely related tick could be appropriately differentiated on gel electrophoresis following PCR (Asadolla et al., 2017). The present investigation was planned to detect the genetic variability through genetic screening using RAPD marker in the

populations of tick species for the first time, from different locations in and around Akola district, of Maharashtra .

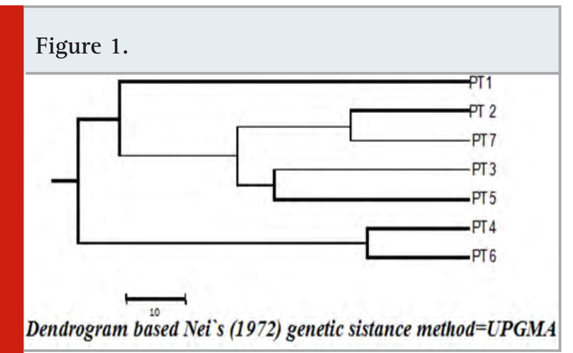
MATERIAL AND METHODS

Experimental ticks were collected from different livestock's located in different places of Akola district Maharashtra and were identified using their standard methods as per taxonomic descriptions provided by Walker et al., (2003). For genetic studies, tissue were prepared and DNA purified from tissue using Promega wizard genomic Kit and Quantified using Nanodrop spectrophotometer (ND 1000, Thermo Corporation, USA) and quality checked on 1% Agarose gel electrophoresis. Gel was visualized using gel documentation system (Bio-Rad Inc., USA). DNA diluted to have 100 ng/µl concentrations and stored at -200C for further use. These DNA samples were processed further for amplification of RAPD. Three RAPD primers, were used which were Pz (CGGCCCGGTA) (Lan et al., 1996) OPJ-01(CCCGGATAA),OPJ-02(CCCGTTGGGA) for the first time in Akola district.RAPD amplification was performed in 25 µl PCR reaction using Kapa biosystems PCR kit. The reaction constitutes 17.8 µl Nuclease free water

2.5 µl 10X PCR buffer,
0.5 µl MgCl2,
2.0 µl 2.5mM DNTPs,
2 µl 10mM primer (PZ and OPJ1) and
0.2 µl taq polymerase (5 units/µl)
and 2 µl of 100ng/ µl of template DNA.

The PCR thermal cycle conditions include an initial denaturation step of 5 min at 95^{0}C, 35 cycles of 30 sec at 95^{0}C, 40 sec at 36^{0}C, and 1 min at 72^{0}C, with a final extension at 72^{0}C for 10 min. Amplified PCR products were visualized on 2% agarose gel and were photographed under UV illumination with gel documentation system.

Figure 1.



Dendrogram based Nei`s (1972) genetic sistance method=UPGMA

RESULTS AND DISCUSSION

Three RAPD primers, were used which were Pz (CGGCCCGGTA) (Lan et al., 1996) OPJ-01(CCCGGATAA),OPJ-02(CCCGTTGGGA). The RAPD banding profiles were computed and analyzed based on presence or absence of bands. Molecular size of amplified bands were estimated by comparing with known molecular weight marker (1kb bp Universal DNA ladder, KAPA Biosystems, USA) The RAPD finger printing patterns for adult of Hyalomma a. anatolicum, Hyalomma marginatum issaci, Hyaloma hussaini, R.Boophilus microplus Rh.Annulatus. Rh. Haemaphysaloides, Haemaphysalis bispinosa obtained with Pz primer are shown in figure Plate.1. The pattern of bands obtained for each sample was species specific. All amplifications were repeated twice and only reproducible bands were considered for scoring. No amplifications were found from OPJ2 primer fragments. According to the Photo plate 1, the gel image under gel-documentation showed the amplification from PZ primers, the same bands were recorded in PT2 (Hylomma. issaci) and PT5(Rh. annulatus) giving the close genetic pattern in both the species. Some similar amplification are specifically observed in PT1 (Rh.Boophilus microplus), PT3 (Hyalomma a. anatolicum), PT4 (Hyalomma hussaini) andPT7 (Hylomma bispinosa).

In PT7 Hylomma bispinosa species we found some different amplification from the other ticks species. The same gel image after amplification with the OPJ1 primer showed that, PT1(Rh. Boophilus microplus), PT2 (Hyalomma marginatum isaaci) and PT5 (Rh.Annulatus) giving maximum similar amplifications proved that these 3 species of ticks are genetically very close to each other for the genetic fragments of OPJ1 primer. Amplification for PT3, PT4, PT6 and PT7 these four species to be observed genetically close banding patterns with OPJ1 primer. In species PT6 and PT7 some more amplification are observed. Ticks are typically recognized with microscopic and morphometric investigation. But, there are many difficulties

Table 1. Nei's Original Measures of Genetic Identity and Genetic distance

(Genetic distance ↓ below diagonal; Genetic identity ↑ above diagonal)

Pop ID	PT1	PT2	PT3	PT4	PT5	PT6	PT7
PT1	****	0.3536	0.4472	0.1768	0.2887	0.25	0.189
PT2	1.0397	****	0.4743	0.25	0.4082	0.3536	0.6682
PT3	0.8047	0.7458	****	0.3162	0.5164	0.2236	0.5071
PT4	1.7329	1.3863	1.1513	****	0.2041	0.7071	0.2673
PT5	1.2425	0.8959	0.6609	1.589	****	0.2887	0.4364
PT6	1.3863	1.0397	1.4979	0.3466	1.2425	****	0.378
PT7	1.6661	0.4032	0.6791	1.3195	0.8291	0.973	****

linked with accurate tick identification (Poucher et al., 1999), as separate keys must be used for different developmental stages: larvae, nymphet, and female and male adult forms. However, these difficulties could be resolved by using molecular genetic markers-based keys (Poucher et al., 1999). The molecular technique, generate the potential to explore DNA at the individual base pair. This is proved to be a much more straight-way of measuring and determining the genetic variation within and between species (Hoy, 1994). RAPD methods were utilized to generate genetic linkage map for the I. scapularis ticks (Ullman et al., 2003). Yang et al. (2004) used RAPD methods in exploring genetic distance of 7 species of Ixodidae ticks which all showed their specific DNA band. The study by Mohammed and Enan (2018) too used the molecular method and demonstrated that ticks in the UAE are very similar at the genetic level. Thus, it was concluded that RAPD could identified, species of Ixodidae ticks. Different species may have different annealing sites and thus give different patterns of bands (Williams et al., 1990).

Lan et al., (1996) reported that at 12 and 13 bands could obtain for H. asialicum and Boophilus microplus using Pz primer, respectively. In our study, similar results were recorded that from both the primers PZ and OPJ1, annealing at 4000 bp base pair was significantly observed. There were not many reports available with reference to ticks population and genetic diversity studies using molecular markers except a few studies which were restricted only to rare species of ticks (Yang et al., 2004, Len et al.1996 ; Hernandez et al.,1998) in the present study, the genetic variation among seven isolated of the different species of ticks within various location of Akola district, Maharashtra in India was documented i.e. three random primers was used to analyze the close relationship. In this study described here, we could successfully use the Pz and OPJ1 primer fragments for the 7 Indian hard tick species viz; Hyalomma a .anatolicum, Hyalomma marginatum issaci, Hyaloma hussaini, Rh. Boophilus microplus Rh. Annulatus. Rh. Haemaphysaloides, Haemaphysalis bispinosa obtained. Based on the reproducible patterns recorded for 7 species of ticks, the primers were recorded to be specific for the species. Thus, the seven studied species are having the particular genetic makeup comparable with those found elsewhere in the world. Thus the molecular taxonomy may be better than only morphological identification especially for differentiation of closely related tick species, as mentioned by Asadolla et al., (2017).

According to Mohammed and Ensan (2018) though, more comprehensive studies are needed, the primers should be valuable candidates for detection of the ticks. Our study though at preliminary level, suggest the prospective of RAPD-PCR for distinguishing ticks at the species level as well as in our better understanding; this is the first report of primers that can be used for the confirmation of tick species, in Study area. Additional study for searching intraspecific variations in specific geographical populations of the ticks from the study area will be tried in future.

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Aqueous Extract of *Carica papaya* Leaf Elicits the Production of TNF-α and Modulates the Expression of cell Surface Receptors in Tumor-Associated Macrophages

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ABSTRACT

Carica papaya leaf has widely been used for its medicinal properties; includes anti-cancer, immunomodulatory, and wound healing. However, its molecular mechanisms of enhancing the immune function remain to be unexplored. In the present study, we investigated the effect of aqueous leaf extract of *Carica papaya* on the production of non-specific effectors molecules and the expression of cell surface receptor by tumor-associated macrophages (TAMs) isolated from Dalton's lymphoma bearing mice. Nitric oxide production was measured by Griess reagent and release of tumor necrosis factor (TNF-α), IL-6 was determined by ELIZA assay. In addition, FACS analysis used to evaluate the expression of cell surface receptor molecules on TAMs. Here, we found that CPE treatment significantly enhanced the production of effectors molecules nitric oxide (NO), hydrogen peroxide (H_2O_2), tumor necrosis factor-α (TNF-α) and IL-6 in TAMs. Furthermore, CPE treatments increase the expression of the co-stimulatory molecule (CD80) on tumor-associated macrophages. Thus, our findings suggest that CPE enhanced the anti-tumor response of TAMs by up regulating the production of potent effectors molecules, and modulating the expression of costimulatory cell surface receptor molecule.

KEY WORDS: *CARICA PAPAYA*, CELL SURFACE MOLECULES, IMMUNOMODULATORY, NITRIC OXIDE, TUMOR-ASSOCIATED MACROPHAGES (TAMS).

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INTRODUCTION

Cancer is a type of disease that results from uncontrolled growth and division of cells, causes millions of death regardless many developments made in the field of its diagnosis, treatment, and preventive measures (He et al., 2016 and Qin et al., 2017). Currently, treatments available for cancer are surgery, chemotherapy, radiotherapy, immunotherapy, vaccinations, and combination therapy, where chemotherapy is most common and widely used for the treatment of highly metastatic cancer (Greenwell et al., 2015). However, drugs used during chemotherapy shows several side effects and put the life of patient under threat. Therefore, people are forced to look at alternative treatment options against cancer. Since a long time, plant products have been used to treat many diseases, including asthma, ulcers, eczema, jaundice, malaria, diabetes, helminths infections and fever (Nguyen et al., 2013).

In modern days, it is one of the primary sources of medicinal drugs in developing countries (Greenwell et al., 2015 Hung et al., 2019). Earlier, secondary metabolites like alkaloids, phenolics, flavonoids, carotenoids, tannins, saponins, papain and chymopapain (Pandey et al., 2016) isolated from the tropical plant were used as an anti-inflammatory, immunomodulatory, and antiseptic agent with relatively less or no side effects (Recioet al., 2012). Thus, people have shifted their focus to formulate plant product based potential drugs to cure cancer (Sivarajet al., 2014). *Carica papaya* is a well-known tropical plant containing several bioactive secondary metabolites with immense medicinal value (Khare et al., 2004). Traditionally, people uses different parts of papaya plant, including fruits, seeds, leaves, shoots, and latex to cure various ailments (Nguyen et al., 2013 and Otsukia et al., 2010). The scientific evaluation of papaya plant product show excellent medicinal values, has property to increases antioxidant level, and reduces lipid peroxidation in the blood (Otsukia et al., 2010 and Seigler et al., 2002). It is highly beneficial in wound healing, cardiovascular diseases, dengue fever, cancer, malaria, and hypoglycaemia (Maniyar et al., 2012 and Nunes et al., 2013). Further, strong immunomodulatory, antitumor, anti-inflammatory, antioxidant, and wound healing properties have reported in papaya leaf extract (Imaga, et al., 2013, Gurung et al., 2009 and Anjum et al., 2017).

Figure 1. Macrophages were harvested from DL-bearing mice, adherence purified, and characterized by staining (A) Immunocytochemistry and also characterization by flowcytometry (B) using anti-CD14 antibody

A

B

Cell Numb er

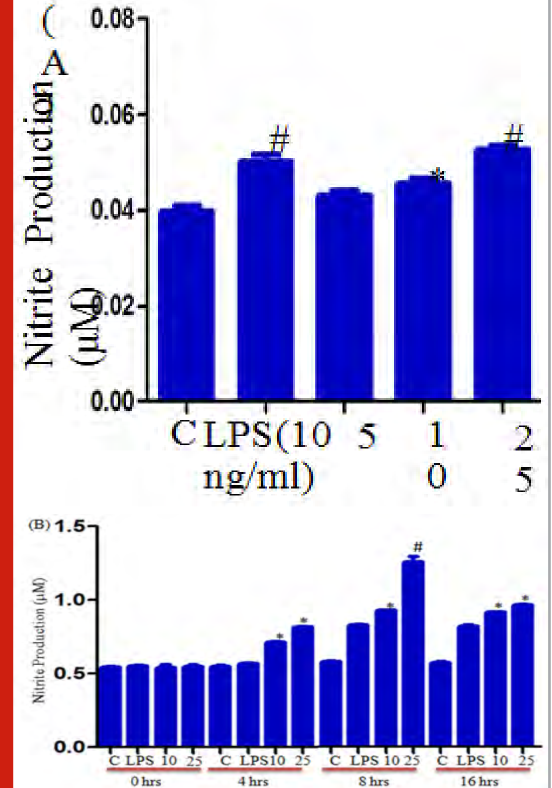
Fluorescence intensity

Recently, immunomodulatory potential of papaya leaf extract reported on several cancer cell lines, but there are very few reports on PBMC (Otsukia et al., 2010), and therefore, required studies in detail. Macrophages are a heterogeneous population of tissue-resident professional phagocytes originated from the terminal differentiation of circulating monocytes (Gordon et al., 2005). They act as a primary line of defence of the host, and mediate innate and adaptive immune response against invading pathogens (Mosser et al., 2008). Stimulation with lipopolysaccharides (LPS), IFN-γ, TNFα, and IL-1β converted resting macrophages into classically activated or M1 phenotype macrophages that shows anti-tumor properties (Edwards et al., 2006).

However, continuous exposure of tumor microenvironment and cytokines like IL-4, or IL-13, IL-10 and TGF β secreted by tumor cells changes M1 macrophages into M2 macrophages which are phenotypically and functionally altered population of normal macrophages and support tumor progression (Goswami et al., 2017). The M2 macrophages or frequently called tumor-associated macrophages (TAMs) are the most abundant cells present in the close vicinity of tumor cells, constituted more than 50 % of the

total tumor mass (Zheng et al., 2017) provide better opportunity to develop a novel anti-cancer therapy by modulating their altered physiology in a tumor-bearing host. In the present study, we have studied immunomodulatory potential of *C. papaya* leaf extract TAMs isolated from Dalton's lymphoma (DL) bearing mice, and found that aqueous papaya leaf extracts significantly induces anti-tumor activities of TAMs and restore its normal morphology to some extent. DL is a type of transplantable T cell lymphoma of mouse origin that mimics human T cells lymphoma, causes death of the host within a short interval of time, and therefore, our finding justify the purpose of the study.

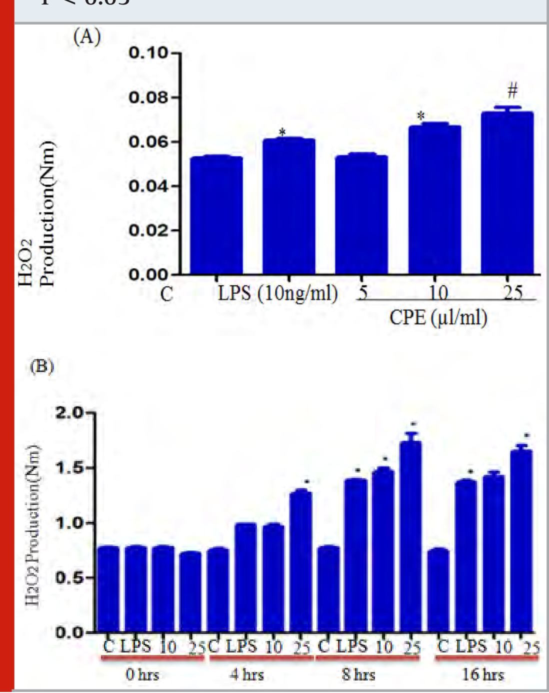
Figure 2. Effect of CPE on nitric oxide production in TAMs. The cells were treated with concentration of (LPS10 ng/ml and CPE 25µl/ml) for 24 h. (A) Nitric oxide production was determined by Griess reagent. Time Kinetics of NO_2 production (B) .Results are representative of 3 independent experiments and analyzed by GraphPad PrismVR 7.0 software using one-way ANOVA with dunnett's test, where all groups were compared with control. # P< 0.0001,* P < 0.05



MATERIAL AND METHODS

Preparation of papaya leaf extract: Collected papaya leaf was wash with autoclaved distilled water. Every 20 grams of leaf powder mixed with 400 ml of water was boiled at 60°C until 12.5% ml water was left as a leaf extract. The extracts were filtered by Whatman filter paper followed by 0.22-micron filter (MILLIPORE), and stored at -20°C. Isolation of tumor-associated macrophage: Tumor-associated macrophages harvested from tumor-bearing mice as described earlier (Gautam et al., 2013). Briefly, a mouse sacrificed by cervical dislocation injected with 2ml chilled PBS in the peritoneal cavity. After intense peritoneal lavaging, peritoneal exudate cells were collected

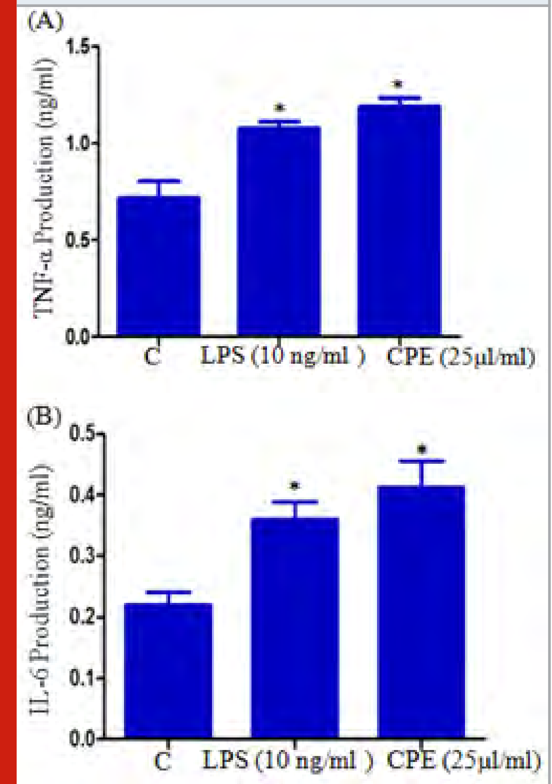
Figure 3. Effect of CPE on the production H_2O_2 in TAMs. TAMs cells were treated for 24 h with concentrations of (LPS 10ng/ml, CPE 25µ/ml). (A) Production of H_2O_2 was determined by ELISA plate reader. (B) Time kinetics of H_2O_2 production. The value represents the H2O2± SEM of three independent experiments in triplicate. # P< 0.0001, * P < 0.05



in a Petri plate and incubated at 37ºC in CO_2 incubator for 2 h. Washed the cells three times with incomplete RPMI 1640 to ensure the removal of all non-adherent cells from the plate after incubation and collected only adherent cells.

Characterization of tumor-associated macrophages: Collected adherent cells were washed in phosphate-buffered saline (PBS) and fixed on Cytospin slide with 4% formaldehyde for 1 h. Block the cells with 0.5% BSA for 1 h at room temperature and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Washed the cells with PBS, stained with anti-CD14FITC antibody (1:100) and incubated overnight at room temperature. After three washing mounted the cells in DABCO and visualized the cells with Nikon E800 upright fluorescence microscope. Purity of macrophages was further confirmed by flow cytometry analysis using CD14PE antibody describe earlier (Lu-Emerson et al., 2013). Nitrite assay for estimation of nitric oxide production:TAMs (1.5x 10_5 cells/well) culture in 200 µl complete RPMI 1640 in a 96 well ELISA plates treated with different concentrations of *Carica papaya* leaf extract CPE (5. 10, 25 µl/ml) and LPS (10ng/ml) as a positive control for 24 h. After desired treatments, the supernatant was harvested and mixed with an equal volume of Griess reagent (sulfanilamide, Naphthalene-ethylene-diaminedihydrochloride, and H_3PO_4) at room temperature for 10 min (Kumar et al., 2006) and the absorbance was measured at 540 nm using a ELISA plate reader. Assay for reactive oxygen intermediate (ROI) production: TAMs cells were seeded and treated with CPE similar to the nitrite assay. After treatment, cells were treated with an equal volume of phenol red solution (140mM NaCl, 10mM K_2HPO_4, 5.5mM dextrose, and 5.5nM horseradish peroxidise), and incubated for 1 hr in CO_2 incubator (Kumar et al., 2006). Subsequently, 1M NaOH added in each wells and took the absorbance at 620 nm on ELISA plate . reader.

Figure 4. Effects of CPE on tumor necrosis factor (TNF)-α, and interleukin (IL-6) in TAMs. TAMs cells were treated with 10 ng/ml LPS and 25 µl/ml CPE for 24 h, and levels of TNF-α (A) and IL-6 (B) were measured by enzyme-linked immunosorbent assay (ELISA) (n = 3). * $P < 0.05$



TNF-α and IL-6 production: 2×10_5 TAMs per well were seeded in a 12-well culture plate and treated with 10 ng/ml LPS or 25µl/ml CPE and incubated for 24 h in a CO_2 incubator. After incubation culture supernatant was collected and measure the level of TNF-α and IL-6 with the help of ELISA assay (Kim et al., 2011), and quantify cytokine concentrations by using standard curves of TNF-α and IL-6.

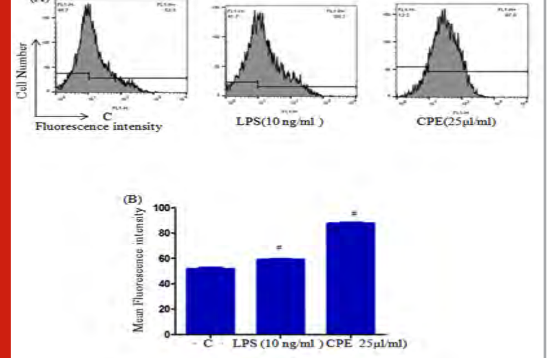
Flow cytometry analysis for cell surface receptor on TAMs: TAMs treated with 10 ng/ml LPS and 25µl/ml CPE incubated in a CO_2 incubator for 24 h. At the end of treatment, cells stained with anti-mouse- CD80 conjugated with FITC and anti-mouse CD14 conjugated with PE (Gautam et al., 2015). After washing with PBS, the cells were re suspended in PBS containing 0.1 % NaN_3, the fluorescence level was measured with the help of flow cytometer (BD Biosciences, Mountain View, CA, USA) LSRII and the data were analyzed by using Flow Jo software.

RESULTS AND DISCUSSION

In the present study, we report that effects of aqueous leaf extract of *Carica papaya* (CPE) on the production of non-specific effector molecules like, NO, H_20_2, and TNF-α, and expression of specific cell surface receptor CD80 on tumor-associated macrophages (TAMs). Macrophages are the part of the innate immune system, and well known to produce non-specific effectors molecules upon stimulation. Here, we isolate and characterized TAMs on the basis of CD 14 expression with the help of flow cytometry (Figure 1A) and found 95 % TAMs after isolation via adherent purification (Figure 1B).CD 14 mainly present on the cell surface on macrophages, and therefore, used as a marker for identifying TAMs (Roszer et al., 2015). It has been reported that activation of macrophages by flavonoid or Polyphenols modulates the production of effector molecules (Yahfoufi et al., 2018).

Nitric oxide is a lipophilic, toxic gaseous molecule, plays vital role in the inhibition of the tumor growth (Lamattina et al., 2003). Several studies have reported that high concentration of NO induces death in the cells, and secreted by macrophages to kill invading pathogens and tumor cells (Rahat et al., 2013). Herein, we examine the effect of CPE on NO production in TAMs and our result shows that CPE treatment significantly increases NO production in TAMs after for 24 h as compared to the control or LPS stimulated cells (Figure 2A). Furthermore, time kinetics study confirmed that the maximum release of NO observed at 8 h after treatment (Figure 2B) corroborate previous finding of Elmowalid and co-workers who reported that an aqueous extract of *Nigella saliva* seed enhances NO production in macrophages (Elmowalid et al., 2013). Further, we investigate the effect of CPE on reactive oxygen intermediate (ROI) production in TAMs and found that CPE treatment increased the H_20_2 production in TAMs as compares to control or LPS stimulated cells. Here, we observed that CPE treatment induced more significant production of H_20_2 compared to LPS treated TAMs (Figure 3A). Time course study confirmed that maximum secretion of H_20_2 from TAMs occur at 8 h of incubation and after that gradually decreased (Figure 3B). Thus, our findings suggest that increased production of RNI, ROI may contribute to reduced tumor growth. Earlier scientists have reported that production of non-specific effector molecules play significant role in enhancing the tumoricidal properties of activated macrophages in vitro and in vivo (Cui et al., 1994).

Figure 5. Effect of CPE on co-stimulatory molecule (CD 80) on TAMs. Cells were treated with concentration of (LPS 10 ng/ml and CPE 25µl/ml). Flowcytometric analysis was carried out to check expression level of cell Surface marker CD 80 molecule. (A) Representing histogram data. (B) The bar graphs represent the mean fluorescence intensity (MFI) ± SEM of positive cells compare to control. #P< 0.0001.



We further measured the effect of CPE on the production of TNF-α and pro-inflammatory cytokineIL-6 in TAMs. CPE Treatment (CPE 25µl/ml) significantly increased the production of TNF-β and IL-6 in TAMs compares to control (Figure 4 A, B). TNF-α is a multifunctional cytokine, which involves in various cellular processes such as cell survival, apoptosis, and inflammation. Several studies reported that LPS could activate J774.1 cells to produce pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β (Hung et al., 2019). Our result indicated that CPE increased the secretion of pro-inflammatory cytokines TNF-α and IL-6 in TAMs. In addition, low levels of MHC-II expression reported on macrophages during tumor progression, as a result it failed to stimulate T cells during tumor progression (Guerriero et al., 2018).However, M1-polarization inducers such as anti-CD40 mAb and IFN-γ are able to up-regulate MHC-II expression

and other co-stimulating factors (e.g. CD80/86) on macrophages to enhance the adaptive immune response of the host required for tumour rejection. Further, we examined the effect of CPE on the expression of co-stimulatory molecule (CD80) on TAMs and shows that CPE treatment enhanced the expression of CD80 molecules significantly (Figure 5). As a result, macrophages provide costimulatory signal for T cell activation. These results collectively suggested that CPE treatment increases the expression non-specific effector molecules and costimulatory molecules on macrophages that in turn increase anti-tumor properties of TAMs and help to attain the M1 phenotype to some extent.

CONCLUSION

The present study shows that *Carica papaya* leaf extract (CPE) positively modulated that production of NO, H_2O_2, TNF-a, and IL-6 production in TAMs. Also, *Carica papaya* leaf extract upregulated the costimulatory receptor CD80 in TAMs. The results suggested that CPE reversing the M2 phenotype polarization to M1 polarization. However, further studies are essential to explore the molecular mechanism induced by CPE in TAMs, which may improve the treatment of cancer.

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Conflict of Interest

Authors declare no conflict of interest in this scientific observation.

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Exosomes as an Exciting Class of a Next-Generation Therapeutic Platform and their Role in Diagnostics

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ABSTRACT

Exosomes are nanovesicles and is a newly emerging field as a novel form of information exchange in various fields of research. Exosomes are a next-generation therapeutic platform and their role in diagnostics acts as a potential vehicle to deliver therapies to cells of the body, as they are rich in cargos like proteins and nucleic acids. Cargo trafficking provides an opportunity to become effective therapeutic reagents for various diseases, like cancer and are also used as biomarkers for the diagnosis. Exosomes are released in biological fluids, such as plasma, serum, urine, and saliva. The present review article provides a comprehensive account on exosome biogenesis, extraction, and their potential use in diagnostics, therapeutic, neurodegenerative disease diagnosis as well as highly precious biomedical tools and their roles in therapeutic vehicles for drug delivery. In future as the field rapidly expands, the different exosome cargos can be used as potential biomarkers which can revolutionize the area of personalized medicine.

KEY WORDS: BIOGENESIS, CARGOS, ESCRT, MVBS, NANOCARRIER.

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INTRODUCTION

Exosomes were first reported by Pan and Johnstone in 1983 at McGill University while culturing sheep reticulocytes during the process of elimination of transferrin (Tfr) receptors, which occur during the maturation process of reticulocytes and erythrocytes. The term exosomes was coined by Trams et al. (1981) who described the release of EVs with 5'-nucleotidase activity from various normal and neoplastic cell lines. Exosomes are disk-shaped extracellular membranous, nano-size vesicles (50–100 nm) that originated from multivesicular bodies (MVBs) (Janas et al., 2016). Exosomes are rich in cargos, which contain proteins, nucleic acids, miRNA, and lipids that directly reflect the metabolic state of the cells from which it originates. Exosomes are efficient messengers of intercellular communication and isolated from body fluids including urine, blood , malignant ascites and cultured supernatants of cell lines, breast milk, cerebrospinal fluid, saliva, amniotic fluid, lymph, bile under healthy and morbid condition. Exosomes are small membrane vesicles that are released by many cell types, including lymphocytes, dendritic cells (DCs) , macrophages, epithelial cells, platelets and tumorigenic cells (Tang et al.,2015, Rashed et al., 2017, Liu et al., 2019).

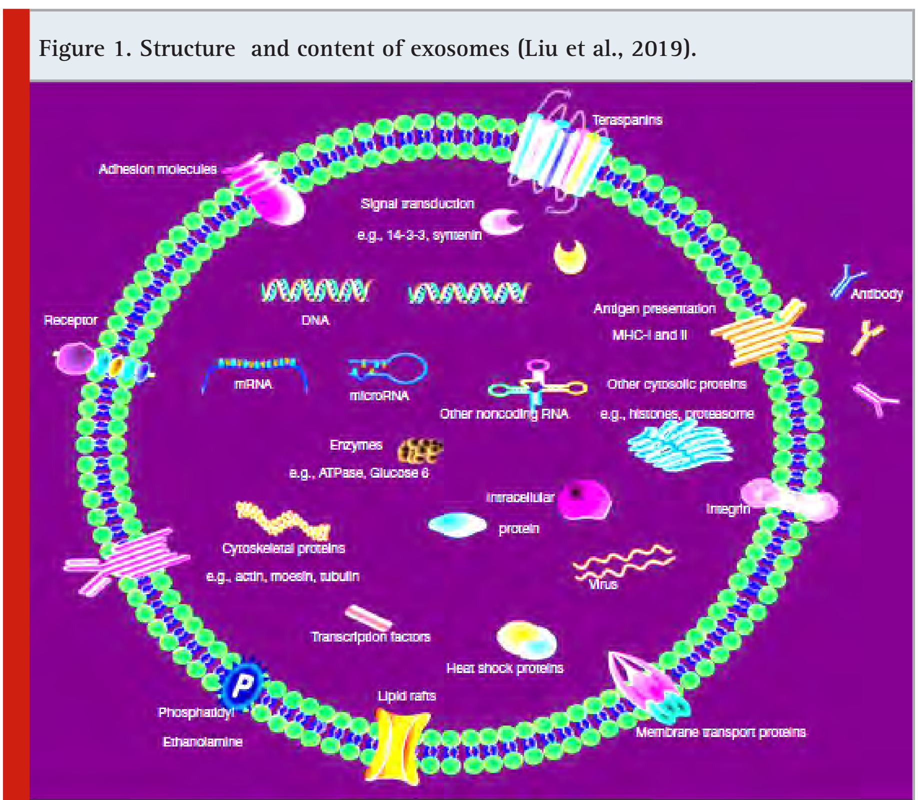
Exosomes formed by membrane invagination of late endosomes, resulting in vesicles that contain cytosolic components and expose the extracellular domain of some plasma membrane receptors at their surface (Huotari and Helenius , 2011). Exosomes remained little studied for the next decade because earlier they were considered as garbage cans which discard unwanted cellular components (Rashed et al., 2017). Exosomes are biomembrane-like vesicles containing specific protein markers of the endosomal pathway. The first classes of nucleic acids identified in exosomes are miR and mRNA, non-coding RNAs (ncRNAs) also the presence of small fragments of single-stranded DNA. The other identified RNA species in exosomes included transfer RNAs (tRNAs), long noncoding RNAs (lncRNAs), and viral RNA. The mRNA and noncoding RNAs such as miRs and lncRNAs in exosomes are functional and can impact the transcriptome of recipient cells. Exosomal contents are depends on their cellular origin and enriched with targeting molecules, membrane trafficking proteins (e.g. Rab proteins, GTPases, annexins and ARF), proteins involved in MVB formation (e.g., ALIX, clathrin and TSG101), cytoskeletal proteins (e.g., actin and tubulin), signal transduction proteins (e.g., protein kinases and heterotrimeric G proteins), chaperones (e.g. small heat shock proteins HSPs), Hsp60, Hsp70, Hsp90, fusion proteins (e.g., tetraspanins, , intergrins and lactadherin), cytoplasmic enzymes (e.g. peroxidases, GAPDH, pyruvate kinases, and lactate dehydrogenase), MHC class I and II proteins , epithelial cell adhesion molecules (EpCAM), and members of the human epidermal receptor (HER) family (Balaj et al.,2011, Kalluri, 2016). Exosomes can be released by all eukaryotic cells and their cargos may greatly differ from each other for function of the originated cell types and their current state (e.g. transformed, differentiated, stimulated, and stressed).

Exosome-specific protein conformation based on cell type or tissue birthplace from which it originates may differ according to the physiological changes and stimulation that the cell underwent. For example, antigen-presenting cell-derived exosomes are enriched in antigen presenting molecules, major histocompatibility class (MHC)-I and -II complexes, as well as co-stimulatory molecules. Tumor-derived exosomes usually contain tumor antigens in addition to certain immunosuppressive proteins such as FasL, TRAIL, or TGF-β. Exosomes also contain proteins involved in cell signaling pathways, such as the Notch ligand Δ- like 4, Wnt-β-catenin signaling proteins and intercellular cell signaling, such as interleukins (Rashed et al., 2017 Zhang et al., 2019a). **Exosome biogenesis:** Biogenesis of exosomes starts in the endosomal system. Exosomes are a class of EVs that originate from the endosome and released from cells when MVBs containing ILVs (Intraluminal vesicles) fuse with the plasma membrane of the cell and release ILVs (which referred as exosomes) into the extracellular environment. The synthesis of exosomes is carried out by two major pathways .This process is highly regulated by multiple signal transduction cascades. Exosomes release from the cell follows the normal exocytosis mechanism characterized

with the vesicular docking and fusion with the aid of SNARE complexes. Both ESCRT-dependent and independent signals have been suggested as determining the sorting of exosomes (Bunggulawa et al., 2018).

ESCRT-dependent mechanisms: ESCRT consists of five distinct protein complexes, namely, ESCRT 0, I, II, and III, AAA ATPase, and Vps4. These complexes drive the inward budding and fission of the membrane to form ILVs sequentially to the cytosolic surface of the endosomal membrane (Rajagopal and Harikumar, 2018). ESCRT-0 is responsible for cargo clustering in a ubiquitin-dependent manner, ESCRT-I and ESCRT-II induce bud formation, ESCRT-III drives vesicle scission, and the accessory proteins (especially the VPS4 ATPase) allow dissociation and recycling of the ESCRT machinery. The ESCRT-III-associated protein ALIX which promote intraluminal budding of vesicles in endosomes and hence exosome biogenesis, upon interaction with syntenin (Kowal et al., 2014). The ESCRT mechanism is initiated by recognition and sequestration of ubiquitinated proteins to specific domains of the endosomal membrane via ubiquitin binding subunits of ESCRT-0 (Zhang et al., 2019a). ESCRT-0 is recruited to pre-MVB endosomes . ESCRT-I and -II also have ubiquitin-interaction domains and sort ubiquitinated cargos at ILV with ESCRT-0. ESCRT-I and ESCRT-II recruit ESCRT-III, which drives the invagination and constriction of the membrane (Mc Gough and Vincent, 2016). The ESCRT complexes accumulate ubiquitinated cargos in exosomes containing both membrane and cytoplasmic proteins. The ESCRT-1 complexes subunit TSG101 is essential for exosomal secretion (Tamai et al., 2010). A study with RNA interference screen targeting, twenty three ESCRT components and associated proteins in HeLa cells reported, ESCRT0 (Hrs, STAM1), ESCRT1 (TSG 101), and the late-acting VPS4 as the main genes responsible for exosomal biogenesis. Some of the components of ESCRT, such as vacuolar protein sorting protein 31 (VPS31), vacuolar protein sorting protein 4B (VPS4B), and TSG101, have been found in endosome-like plasma membrane domains that generate exosomes (Colombo et al., 2013).

Figure 1. Structure and content of exosomes (Liu et al., 2019).



ESCRT-independent mechanisms: The second pathway is independent of the ESCRT machinery and is based on the specific lipid composition of the endosomal membrane. Ceramide was proposed to induce inward curvature of the limiting membrane of MVBs to form ILVs. Raft-based micro domains are present on the limiting plasma membrane of endosomal compartments and contain high amounts of sphingolipids which represent substrates for the neutral sphingomylinase2 (nSMase2). The nSMase2 convert sphingolipids to ceramide at endosomal membrane which induces coalescence of micro domains into larger structures thereby promoting domain induced budding and formation of ILVs. Following the formation of MVBs, they are either destined for the degradative or the secretory pathways, which are governed by Rab GTPases. While Rab7 can mediate the degradation through the fusion of MVBs with lysosomal compartments while other Rab proteins like Rab5b, Rab9a, RAB27a, RAB27b, and Rab35 were reported to be crucial for intracellular MVB trafficking and secretion. The final release of ILVs occurs upon MVB fusion with the cellular plasma membrane, a process which probably mediated, at least in part, by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), like the vesicle-associated membrane protein (VAMP) TI-VAMP/VAMP7.

Once the ILVs are secreted, they are termed exosomes. In tumor cells genotoxic stress leads to an increased activation of p53 transcription factors and to an enhanced p53 and tumor suppressor-activated pathway 6 (TSAP6) expression, which mediates augmented exosome secretion. Release of exosomes can also be induced by diverse signaling pathways including the activation of the Wnt pathway (Dreyer and Baur ,2016).

Cargo sorting: ESCRT proteins, including ESCRT-I, ESCRT-II, and ESCRT-III, are need for cargo selection and the inward budding process (away from the cytoplasm). Tumor cell exosomes have been shown to contain syndecan, syntenin, and ALIX. Down-modulation of these proteins reduced exosomal release, and production of syndecan, syntenin, and ALIX-containing exosomes was dependent on the normal functioning of the ESCRT machinery proteins. The secretion of syntenin into exosomes is driven by syndecan, and this process induces heparin sulfate clustering. Over expression of the enzyme heparinase cleaves the heparin sulfate, causing a noticeable increase in the secretion of exosomes (Rashed et al., 2017).

Exosomal heterogeneity: The molecular composition of exosomes is not only cell-type dependent but can differ even when the exosomes originate from the same parental cells. The subcellular origin of exosomes and donor cell activation status can contribute to their molecular heterogeneity (Ferguson and Nguyen , 2016).

Exosomes web domains: EVpedia and ExoCarta, Vesiclepedia are the web domains in which researchers are able to upload proteomic lists of identified proteins to the database of the exosomes they are working with. This gives an opportunity to create a listing of the most frequently identified proteins generally found in exosomes (Ronquist 2019).

Isolation of Exosomes: The major hurdle in the clinical utilization of exosomes has been the lack of consistent and dependable methods to isolate a pure exosome population (Thery et al., 2006). The differential ultracentrifugation (DC) has been widely used as conventional isolation techniques that separate exosomes and other EVs based on their sizes and buoyant density. Ultracentrifugation is used to pellet crude exosomes. Exosomes were successfully isolated from serum by one-step precipitation method by using commercially available reagents such as ExoQuick (System Biosciences, Palo Alto, CA, USA) as an alternative approach to ultracentrifugation (Rekker et al., 2014).

The affinity capture (AC) methods is used to target established exosomal markers such as CD63, CD81, TSG 101, HSP 70 and Alix, which allows selective capturing of exosome population followed by detection of cancer specific markers. The NTA (Nanoparticle tracking analysis)-based instruments called Nanosight (LM10, LM20, NS200 and NS500) allow exosomes to be counted and sized by combining light microscopy and

the software that tracks Brownian motion of exosomes. NTA method becomes a gold standard to measure the concentration of exosomes (Soung et al., 2017).

Function of Exosomes

The key mechanism by which exosome exert biological function on cell by direct contact between surface molecules of cells and vesicles, endocytosis ,vesicle cell membrane fusion and horizontal transfer of mRNA and miRNA which alters specific gene expression patterns in recipient cells. Biological functions and effects of exosomes promote intercellular communication and are involved in various physiological as well as pathological processes, such as antigen presentation, immune regulation, and tissue development. The nucleic acids in exosomes is important for embryonic and organ development, normal physiology, and various pathologies. Tumor cell-derived exosomes play key role in tumorigenesis, metastasis, and response to therapy through the transfer of oncogenes and onco-miRNAs between cancer cells and the tumor stroma. Exosomes from stimulated blood cells and the vascular endothelium is involved in neurological disorders such as multiple sclerosis, transient ischemic attacks, and antiphospholipid syndrome (Lin et al., 2015).

The molecular composition of exosomes has significant potential as biomarkers for disease diagnosis. Exosomes derived from antigen presenting cells have ability to express major histocompatibility complex (MHC) class I and II molecules on the cell surface, which helps inactivating CD8+ and CD4+ T-cells to induce specific immune responses by carrying prostaglandins, exosomes secreted by platelets are involved in the inflammatory response. Glioblastoma cells also secret exosomes containing mRNA, miRNA and angio genetic proteins to microvascular endothelial cells, stimulating angiogenesis. Exosomes are involved in many biological processes, including the maturation of erythrocytes, the elimination of unnecessary proteins and RNA (Ha et al., 2016). Exosomes biobanking activities and its role in clinical personalized applications rely on discrimination and precise diagnosis, targeted therapies of choice for each patient, dose adjustment methods to optimize the benefit-risk ratio of treatment and biomarkers of efficacy, toxicity, treatment discontinuation, relapse will be beneficial if coordinated with pathologists and clinicians at hospital and health care centers (Mora et al., 2016).

Exosomes in Cancer diagnostics and therapeutics:

Cancer cells derived exosomes have a great potential to serve as a liquid biopsy tool for various diseases. The cargo reflective of genetic or signaling alterations in cancer cells of origin are carry through cancer derived exosomes served as biomarker for early detection of cancer. Exosome based liquid biopsy merits over conventional tissue biopsy because it provides the convenient and non-invasive way of diagnosis over tissue biopsy that requires surgery (Soung et al., 2017). Exosomes can be loaded with different therapeutic drugs, antibodies or RNAi for gene expression, manipulation in order to treat cancer cells in a more efficient manner. Doxorubicin, a chemotherapeutic agent, loaded in breast cancer-derived exosomes (ExoDOX) is more efficient than free doxorubicin in the treatment of breast cancer and ovarian cancer mouse models. Therapeutic approaches that take advantage of exosomes and their characteristics have shown to improve the efficacy of chemotherapy (Bastos et al., 2018).

Exosomal Surface Proteins as Cancer Biomarkers:

Exosomes contain a variety of proteins that reflect their origin and alteration of the parental cells. Based on endosome-based biogenesis pathway, exosome specific protein markers include endosome associated proteins (e.g., small Rab family GTPases, annexins and flotillin), proteins involved in exosome biogenesis (e.g., Alix, Tsg101 and ESCRT complex), tetraspanins (e.g., CD9, CD37, CD53, CD63, CD81 and CD82), heat shock proteins (Hsp70, Hsp90) and epithelial cell adhesion molecules (EpCam) . The proteomic analyses of cancer-derived exosomes led to the identification of potential exosomal markers to serve as a liquid biopsy in breast, prostate, pancreatic, ovarian, colorectal cancers and glioblastoma (Soung et al., 2017). Tumor-derived exosomes (TEX) are important intercellular messengers that contribute to tumorigenesis

and metastasis through a variety of mechanisms such as immunosuppression and metabolic reprogramming that generate a pre-metastatic niche favorable to tumor progression (Tung et al., 2019).

The Role of Exosomes in Infectious Diseases: Pathogen-released exosomes apart from the widely studied immunomodulatory effects are known to carry specific virulence factors, such as proteins, mRNA, and miRNA, which contribute to spread the infection. It is known that even parasitic trematodes and nematodes release exosomes as an immunomodulatory mechanism (De Toro et al., 2015). Exosomes play an important role not only in the process of infection by pathogens but also in anti-infection. Exosomes mediate infection through transferring pathogen-related molecules (pathogenic genes and proteins) or even the entire pathogens. Exosomes can be either directly infectious, alter nuclear gene expression, or mediate toxic reactions, participating in pathogen immune escape mechanisms; inhibiting immune responses by inducing immune cell apoptosis. Exosomes can play anti-infective roles by inhibiting pathogen proliferation and infection directly inducing immune responses (Zhang et al., 2018).

Exosomes in drug delivery: The exosome-based drug delivery gained momentum after successful demonstration of nerve cell-derived exosomes for increasing immunity in cancer patients and for targeting cancer genes. Other cell types that have been used as exosome factories include stem cells, human and mouse cancer cells, etc. Exosomes from these cells were shown to deliver small RNAs for gene silencing and small drugs (e.g., doxorubicin and curcumin). The concern about safety and cancer-stimulating properties of exosomes derived from the stem cells and cancer cells are much more. Exosome-like particles and lipids derived from fruits (e.g., grapes and grapefruit) were recently examined as an alternative drug carrier. Vesicles isolated from the bovine milk are mostly used for drug delivery works. It is considered as a good exosomal source for the drug delivery purposes because of no adverse immune and inflammatory effects. The milk exosomes represent the most useful drug-carriers that can be exploited to deliver all kinds of agents ranging from small molecule drugs to nucleic acids to proteins (Munagala et al., 2016). Exosomes are used in packaging of drugs instead of using synthetic nanoparticles. Exosomes are promising agents for drug delivery because of low immunogenicity, innate stability, and high delivery efficiency. Peptide-conjugated exosomes loaded with curcumin as a drug has been proved as a good system for the effective drug delivery for brain ischemia (Tian et al., 2018).

Exosomes in degenerative disease: Neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies or fronto-temporal dementia, show clinical and pathological overlaps and difficulty in specific and differential diagnosis. Now a days biomarkers for each of these types of dementia have been investigated in the cerebrospinal fluid or blood (Gamez-Valero et al., 2019). A common pathological feature of many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS or prion diseases, is the abnormal deposition of proteins in the brain with implicated exosome function.

Increasing evidence implicates exosomes in synaptic function, exosomal transfer of proteins, mRNAs, and miRNAs plays a role in synaptopathies an important mechanism by which NPCs may propagate some of their immune modulatory functions (Janas et al., 2016). Exosomes are proposed to be involved in the spread of 'toxic' proteins in neurodegenerative disorders. Mutated or misfolded proteins serve as template for the formation of oligomers, leading to neuronal toxicity. Removal of these accumulated proteins is thought to occur by processing them through endosomal pathway, which either leads to lysosomal degradation or incorporation into MVBs and release into the extracellular space as exosomes. This phenomenon was reported in the case of Aβ peptide in Alzheimer's disease. Exosomal release of Aβ is likely a mechanism to remove excess intracellular Aβ Exosomes appear to be a major vehicle that shuttles amyloids out of the cell and act as seeds for plaque formation. Neuron-derived exosomes containing normal Aβ

levels and neuroprotective factors may act as scavengers for synaptotoxin Aβ species, thereby mediating neuroprotection (Janas et al., 2016).

Hamlett et al. (2018) reported neuron-derived exosomes, contain elevated levels of amyloid-beta peptides and phosphorylated-Tau that could indicate a preclinical AD (Alzheimer's disease) phase in people with DS (Dejerine-sottas disease) starting in childhood. The inhibition of exosome secretion is associated with reduced AD(Alzheimer's disease)-like pathology in a transgenic mouse model of AD. Dinkins et al. (2014) found exosomal proteins accumulating in the plaques of AD patient brains, and able to reduce amyloid plaque load in AD brains *in vivo* by decreased secretion of exosomes, achieved by inhibition of nSMase2. Recently, exosomes emerged as novel biological source with increasing interest for age-related neurodegenerative disease biomarkers (D'Anca et al., 2019).

Targeted exosome - drug addiction: Cell derived exosomes have been demonstrated to be efficient carriers of small RNAs to neighbouring or distant cells as carriers for gene therapy over other artificial delivery tools. Modified exosomes expressing the neuron-specific rabies viral glycoprotein (RVG) peptide on the membrane surface to deliver opioid receptor mu (MOR) siRNA into the brain to treat morphine addiction. MOR siRNA delivered by the RVG exosomes strongly result in inhibition of morphine relapse via the down-regulation of MOR expression levels. This provides a brand new strategy to treat drug relapse and diseases of the central nervous system (Liu et al., 2015).

Regenerative and Anti-inflammatory Therapies: Mesenchymal stem cells (MSCs) which are well known to possess anti-inflammatory and regenerative effects are the most commonly used source of exosomes. It showed that exosomes secreted from iPS cells, embryonic stem cells, and cardiac progenitor cells have therapeutic effects similar to MSC-derived exosomes (Khan et al., 2015, Vrijsen et al., 2016).

Exosomes in diagnostics: Exosomal contents, nucleic acids, proteins and lipids, are altered during disease conditions. Therefore these are an attractive target for clinical diagnostics and biomarker discovery. Exosomes can be isolated non-invasively from easily accessible biological fluids including urine, blood and saliva. Non-invasive approach allows for early diagnosis of disease, which is importance in diseases of the CNS. The exosomal contents are protected inside a membranous structure, which gives an advantage over conventional specimens as the potential biomarkers are protected from degradation. These are highly stable, making their clinical use feasible as samples can be stored for prolonged periods before analysis. Exosomes can be traced to their origin as they express surface markers related to their cellular origin (Kanninen et al., 2016). Exosomes from human serum are employed to detect HIV-positive patients. At present this test is commercialized (Vlassov et al., 2012). Thirty three unique proteins of *Mycobacterium tuberculosis* were identified from exosomes isolated from human serum that may serve as biomarkers for persistent active and latent tuberculosis (Kruh-Garcia et al., 2014).

Exosomes in Angiogenesis: Angiogenesis is a major process which regulates nutrient availability of fast growing solid tumors. Tetraspanins are key players in the process of angiogenesis . Exosomal interaction and uptake of endothelial cells (ECs) will induce angiogenesis with the incorporation of vesicular cargos such as tetraspanin 8 and CD106 and 49d, which can activate vascular endothelial growth factors (VEGFs). Exosomes released from the endothelial progenitor cells interact with mature endothelial cells and its cargo integration triggers AKT signaling, resulting in angiogenesis (Rajagopal and Harikumar, 2018). Neoangiogenesis is as entry point for metastatic cells into systemic circulation. Neoangiogenesis has to be stimulated to avoid cancer necrosis. Cancer-associated fibroblasts stimulate neoangiogenesis via secretion of exosomal SDF-1 (stromal cell derived factor-1). The released SDF-1 is responsible for the recruitment of endothelial progenitor cells by chemotaxis .The same result was found after treatment of umbilical cord mesenchymal stem cells with gastric-cancer cells derived exosomes or with exosomes from hepatic cancer cells (Steinbichler et al., 2017). Theragnosis is a concept

in next-generation medicine that simultaneously combines accurate diagnostics with therapeutic effects. Molecular components in exosomes have been found to be related to certain diseases and treatment responses, indicating that they may have applications in diagnosis via molecular imaging and biomarker detection (Kim et al., 2018).

The role of exosomes in pregnancy: Exosomes are produced and secreted by syncytiotrophoblast of the human placenta continuously and constitutively in the maternal bloodstream. This exhibits a redundant number of mechanisms that inhibit the function of the maternal immune system during pregnancy and promote the survival of the fetus. The role of immunosuppressive placental exosomes during normal pregnancy is clear, the contribution of exosomes in pathological pregnancies and related diseases, such as recurrent abortions and infertility, need a more profound evaluation. The knowledge derived in these areas will set up possibilities for novel, exosome based treatments of infertility and pregnancy failure (De Toro et al., 2015).

Cancer Vaccines: Exosomes in immunotherapy could form a viable basis for the development of novel cancer vaccines, via antigen-presenting cell technology, to prime the immune system to recognize and kill cancer cells (Tan et al., 2010). Exosomes derived from professional antigen presenting cells enable them to activate directly CD8+ and CD4+ T-cells inducing a strong immunogenic response. Exosomes can activate T-cells either by a direct antigen presentation or by an indirect presentation through transfer of antigenic peptides to APCs. Tumor-derived exosomes express tumor antigens that can activate DCs, thereby priming the immune system to recognize and promote a specific cytotoxic response with a higher immunogenicity than that accomplished by tumor cell lysates or soluble antigens when used as vaccines (De Toro et al., 2015). Serum-derived exosomes from pigs are revealed for its use for the development of the vaccine against porcine reproductive and respiratory virus (PRRSV) (Montaner-Tarbes et al., 2016). Exosomes as immune modulators, may be either immune activating or immunosuppressive agents. Exosomes were proposed as acellular antigens for the development of vaccines against either infectious diseases or tumors. Ascetic cell-derived exosomes (AEX) taken from peritoneal cavity fluid in cancer patients have been shown to cause tumor cell lysis by inducing dendritic cells to prime T lymphocytes via an MHC I dependent pathway to kill cancer cells. This also triggers the release of IFN-γ by peripheral blood lymphocytes in *vitro* experimental models (Andre et al., 2002).Exosome-based cancer immunotherapy is an attractive approach against cancer as tumor derived exosomes carrying tumor associated antigens are reported to recruit the immune responses (Shao et al., 2016). Exosomes derived from dendritic cells (DCs) may be useful as anticancer vaccines because of the nature of DCs as antigen-presenting cells (APCs). Major histocompatibility complex (MHC)-I, MHC-II, and co-stimulating factor such as CD86 are expressed on the surface of DC-derived exosomes (Yamashita et al., 2018).

Future Perspectives: Exosomes offer a promising tool for the invasive diagnosis, monitoring cancer, diagnosis in tumero genic studies and neuro generative disorders. The great potential of this 'platform' technology has several advantages over other nano carrier based therapies and offers simplicity and versatility approach for delivery of drugs. The future prospects for exosomes as therapeutic agents have lots of potential, as new biomarkers can be identified and use them in diagnostic applications. The available information on exosome biogenesis and functions can be used as significant opportunities to manipulate their properties, composition and cell interactions to further advance improves their therapeutic platform and drug delivery potential. Recent advances in using exosomes as biomarkers for disease detection and as drug/gene delivery systems have been stimulating (Li et al., 2019). The combination of exosomes with different therapeutic cargoes often makes them immunogenic based on the nature of parental donor cells, and therefore, further studies are needed to describe immunogenic reactions after administration. Nanotechnology coupled with engineered exosomes is emerging as new and novel approach for cancer vaccine development

(Tan et al., 2010). The exosomal miRNA has potential to explore as non invasive biomarker or to indicate disease status of patient on the basis of difference in amount and composition of exosomal miRNA from healthy and diseased patient (Zhang et al., 2015). Further exploration about the structure and function of exosomes to find ways of developing and standardizing appropriate methods to modify exosome contents in a loading process and drug delivery will contribute to the therapeutic, and clinical application of exosomes.

The progress achieved in the evolving field of exosomes in recent years has been exciting and quick (Wang et al., 2019). The role of tumor-derived exosomes in cancer progression and use of exosomes for cancer diagnosis and treatment may change effectiveness of cancer treatment. This can lead to personalized medicine and influence the direction of future research on exosomes. Exosomes have the inherent ability to cross the BBB, the major obstacle for brain targeting. Exosomes can be excellent vehicles for drugs, including small molecules as well as bio macromolecules to treat CNS diseases (Zhang et al., 2019 b).

CONCLUSION

The area of research on utilization of exosomes and their cargos is a novel and rapidly growing field with interest. Exosomes are cell-derived membrane vesicles secreted by different cell types and harbor verity of molecular constituents and proteins which are present in various body fluids. The interest in exosomes has increased exponentially mainly because of the important potential application in biological, pathological and physiological function. This holds a great promise as novel biomarker, in clinical diagnosis ,prognostic indicators, development of therapeutics, drug delivery and vaccines. In future a more thorough understanding and analysis of exosomes and their components will enable the development of safer and more effective exosomes with defined structures and functions.

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Pharmaceutical Activities of Certain Phytochemicals from the Leaf and stem of *Hydnocarpus macrocarpus*

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ABSTRACT

The present study evaluates the preliminary phytochemical profile of the leaf and stem of *Hydnocarpus macrocarpus* with reference to their antifungal activity. Antifungal activity was evaluated using Agar-Well Diffusion Method and the preliminary phytochemical screening method was done using Pew's tests, Alkaline test, Ninhydrin test, Xantho proteins test, Conc. H2SO4 test, Xanthoprotetic test, Biuret test, Mayer's test, Hager's test, Wagner's test Salkowski's Test, Keller killiani Test, Glycoside Test, Keller kiliani Test Foam Test, Test for Coumarins, Braymer's Test, Potassium dichromate test and Ferric chloride test. Antifungal activity of leaf and stem was tested with three fungal pathogens. It revealed that the leaf and stem extracts of acetone and methanol showed the highest activity against the pathogens *Aspergillus niger* and *Mucor indicus*. Phytochemical screening showed the presence of alkaloids, carbohydrates, flavonoids, steroids, glycosides, phenols, proteins, tannis, saponins, terpenoids, and fixed oil in the leaf and stem of *H. macrocarpus*. The result obtained in the in vitro methods suggest that *H. macrocarpus* stem and leaves may be administered for their phytochemical and antifungal activity.

KEY WORDS: *HYDNOCARPUS MACROCARPUS*, PHYTOCHEMICAL PROFILE, ANTIFUNGAL ACTIVITY.

ARTICLE INFORMATION

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INTRODUCTION

Medicinal plant products possess unique chemical diversity because of diverse bioactive compounds in them. Constant uses of herbals have led to the effective drug discovery for the treatment of human diseases, (Galm and Shen, 2007, Afolayan 2013 Ganesh et al., 2019). *H. macrocarpus* is a huge tree, evergreen. The tree is probably harvested from the wild for the gifted seeds, known for medicinal uses. It is an endangered tree confined to southwest India. The prime habitat of the tree has been severely damaged because of anthropogenic pressure as example the Kodayar Hydroelectric Project the establishment of plantation crops in Tamilnadu, South India. Outlying tree populations exist further north towards the Anamalais. The fruits with medicinal properties are stimulants of respiration and enable digestion. In excess, however, they can cause respiratory failure and even death. The leaf and stem also showed high medicinal values inhibiting microbes, both Gram positive and negative, (Ganesh et al 2019). Botanical descriptions: Trees up to 12 m tall.

Bark greyish brown, lenticellate; blaze cream. Branches with architecture of "Aubreville model" branchlet sterete with fallen leaf scars, lenticellate, rusty or greyish stellatetomentose. Leaves simple, alternate, spiral, clustered at twig ends; petiole 2.5 cm; lamina 7.5-10 × 5-7.5 cm, elliptic, folded boat-shaped, apexacute to shortly acuminate, base acute, with very shallow serrations. Inflorescence in racemes, 5-10 cm long, 10 flowered; pedicels 2 cm long, tomentose; petals cream, laciniate; anthers awned Distribution: Southern Peninsula (Western Ghats). Corner, Gard. Bull. Straits Settlem.10: 319, 325. 1939; Matthew, III. FI. Palni hills t. 81. 1996. As said earlier leaves and fruits in *H. macrocarpus* are used as stimulants for respiration and as agents of improved digestion. It is also claimed to be of benefit in the treatment of cancer. Seed powder is used against constipation, irritation and other skin diseases. The oil of *H. macrocarpus* plays the greatest role in medicinal field, not only the seed but also the leaf and stem as an effective antibiotic, (Ganesh et al., 2019). The investigation of the paper confirms the reported validity of *H. macrocarpus* in the plant organs of leaf and stem as ant microbial in nature.

MATERIALS AND METHODS

Collection and Authentication: The plant was collected from the Western Ghats, Kerala, India, during April 2017. The plant was identified by Dr. S. John Britto, Director and Head, The Rapinat Herbarium and Center for Molecular Systematics St. Joseph's College (Autonomous) Tiruchirappalli, India. The voucher specimen RHT: 68237 were deposited at The Rapinat Herbarium. Extraction of plant material: Leaves were air dried under shade at room temperature, ground with electric grinder into fine powder and stored in air tight container for further use. 10 grams of powdered sample mixed in 150 ml of solvents (i.e. methanol, ethanol, acetone, Chloroform, Petroleum either and water) for extraction, was kept in rotary shaker for three days at room temperature. The extracts were filtered by using Whatmann filter paper then air dried and stored for further usage. The crude extracts were further re-suspended in 1 ml of respective solvents for the investigation of phytochemical and antibacterial activities.

Phytochemical screening and Antifungal activity: For alkaloids: Wagner's Test: 2 ml of extract was treated with few drops Waner's reagent. Formation of reddish brown precipitate indicated the presence of alkaloids. Hager's Test: 2 ml of extract was treated with few drops of Hager's reagent (saturated solution of picric acid). Formation of yellow color precipitate signified positive result. Mayer's Test: 2 ml of extract was treated with few drops of Mayer's reagent. Formation of cream precipitate indicated the presence of alkaloids. Test for proteins Biuret Test: 2 ml of extract was treated with 2 ml 5%NaOH and 2 ml 1% $CuSO_4$ solutions. Violet or purple coloration indicated presence of proteins and free amino acids. Xanthoprotetic Test: 2 ml of extract was treated with few drops of concentrated HNO_3. Formation of yellow color indicated the presence of proteins. Conc. H_2SO_4 Test: 2 ml extract was treated with few drops of conc. H_2SO_4. Formation of white precipitate indicated the presence of proteins. Xantho proteins Test: 2 ml of extract was treated with few drops of conc. HNO_3 and NH_3

solution. Formation of reddish orange precipitate indicated the presence of xantho proteins.

Test for amino acids: Ninhydrin test: 2 ml of extract was treated with 1ml of freshly prepared 0.25% ninhydrin reagent and boiled for few minutes. Formation of blue color indicated the presence of amino acids. Test for flavonoids: Alkaline Test: 2-3 ml of extract was treated with few drops of NaOH solution. Formation of intense yellow color which turned colorless on addition of few drops of dilute HCl. Pew's tests: 2-3 ml of extract was treated with zinc powder in a test tube, followed by drop wise addition of conc. HCl. Formation of purple, red or cherry color indicates the presence of flavonoids. Lead acetate test: 1 ml extract was treated with 1 ml 10% lead acetate (Pb(OAc)4) solution. Formation of yellow Color precipitate indicated the presence of flavonoid. Conc.H2SO4 test: 5ml of dilute ammonia solution was added to the extract followed by conc.H2SO4. Yellow color indicated the presence of flavonoids. Test for fixed oils CuSO4 test: 2 ml of extract was treated with 1 ml of 1%CuSO4 solution and 10% NaOH solution. Blue coloration indicated the presence of fixed oils.

Test for phenols and tannins Ferric chloride test: 2 ml of extract was treated 2-3 drops of 5% ferric chloride solution. Formation of bluish-black color showed presence of phenols and black color tannins.Potassium dichromate test: 2 ml of extract was treated with 5% potassium dichromate solution. Positive result was confirmed by a formation of brown precipitate (for phenol). Braymer's Test: 2 ml of extract was treated with 2 mlH2O and followed with 2-3 drops of FeCl3 (5%). Green precipitate proved presence of tannins.Test for Coumarins: 2 ml of extract was treated with 3ml of 10% NaOH solution. Yellow coloration indicated the presence of coumarins. Test for saponins Foam Test: 2 ml extract was diluted with 10 ml of distilled water and warmed gently. It was shaken for 5 minutes. Persistent froth indicated the presence of saponins. The same extract was added with few drops of olive oil. Formation of a soluble emulsion, confirmed the presence of saponins.

Test for Glycosides: Keller kiliani Test (*Test for cardiac glycoside*): 2 ml extract was treated with 1 ml glacial acetic acid, one drop 5% FeCl3 and 1 ml conc. H2SO4. A brown ring of the interface indicated the presence of cardiac glycosides. Glycoside Test: Small amount of extract was treated with1 ml water and shaken well. Then aqueous NaOH was added. The appearance of yellow color indicated the presence of glycosides. Test for sterols Salkowski's Test: 2 ml of extract was treated with 2 ml chloroform and 2 ml conc. H2SO4. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence indicated the presence of sterols. Keller killiani Test: (*Test for cardiac glycoside*): 2 ml extract was treated with 1 ml glacial acetic acid, one drop. 5% FeCl3 and 1 ml conc. H2SO4. A brown ring of the interface indicated the presence of cardiac glycosides. Test for Terpenoids Salkowski's Test: 2 ml of chloroform and 1 ml ofconc.H2SO4 was added to 1 ml of extract and observed for reddish brown color that indicated the presence of terpenoids. Antifungal activity: The antifungal assay was carried out in the Tropical Institute of Ecological Sciences, Kottayam, affiliated to Mahatma Gandhi University, Kerala. Selection of Fungal organisms.

The pathogenic fungal species were obtained from Tropical Institute of Ecological Sciences, Kottayam, Kerala. *Aspergillus fumigate*, *Aspergillus niger* and *Mucor sp*. Agar-Well Diffusion Method (Murray et al., 1995; Olurinola,1996). Protocol for the antifungal activity was adopted from Murray (1995) in which ethanolic extracts of twenty ethnomedicinal plants were investigated earlier. Olurinola later modified the Method in 1996. Preparation of Fungal Inoculums: The inoculum was prepared from 5 – 6 days old culture grown on Potato Dextrose Agar Medium (PDA). Petri dishes were flooded with distilled water and conidia were scraped using a sterile spatula to release the spores. The spore density of each fungus adjusted by spectrophotometer (A595 nm) to get a final concentration of approximately 105 spores/ml (Mahesh & Satish, 2008).Culture Media Used> The Potato Dextrose Agar (PDA) medium was used for the antifungal studies. It consisted of following composition (for 1000 ml): -

Table 1. Phytochemicals of H. macrocarpus leaf and stem

S.No.	Phytochemical constituents	Acetone	Aqueous	Extracts Chloroform		Ethanol		Methanol				Petroleum ether	
		L	S	L	S	L	S	L	S	L	S	L	S
1.	Test for Alkaloids												
	Hager's Test	++	+	++	++	-	-	++	-	++	++	+	-
	Mayer's Test	++	++	+++	++	-	-	++	-	++	++	-	-
	Wagner's Test	++	++	-	++	-	-	++	+	++	++	-	+
2.	Test for Carbohydrates												
	Molisch's Test	+	++	++	+	-	-	+	++	+	++	-	-
	Fehling test	+	-	+	-	-	-	-	+	-	+	-	-
	Benedict's Test	+	+	+	-	-	-	+	+	+	+	-	-
3.	Test for Flavanoids												
	Alkaline Test	+	+	+	++	-	-	+	-	+	+	-	-
	Conc.H_2SO_4 Test	+	+	++	+	-	-	+	+	++	+	-	-
	Pew's Test	++	++	++	-	-	-	+	++	++	++	-	-
	Lead acetate	++	+	++	+	-	-	++	+	++	+	-	-
4.	Test for fixed oils												
	$CuSO_4$ Test	++	+++	++	++	+	+	++	++	+	++	+	+
5.	Test for Phenols												
	Ferric chloride Test	+++	+	++	-	-	-	++	++	++	++	-	-
	Potassium Dichromate Test	-	+	-	-	-	-	-	++	-	++	-	-
6.	Test for Tannins												
	Ferric chloride Test	-	+	+	-	-	-	+	+	+	+	-	-
	Braymer's Test	+	+	+	-	-	-	+	+	+	+	-	-
7.	Test for saponins												
	Foam Test	++	+	++	+	-	-	++	+	+	++	+	-
8.	Test for Glycosides												
	Keller kiliani Test	++	++	++	+	-	+	++	++	++	++	-	-
	Glycoside Test	+	+	+	+	-	+	+	+	+	+	-	-
9.	Test for Coumarins												
	10%NaOH Test	+	+	+	+	-	+	+	+	+	+	+	-
10.	Test for Sterols												
	Salkowshi's Test	+	++	+	-	+	+	+	++	+	++	-	-
	Keller killiani Test	++	++	++	+	-	+	++	++	++	++	-	-
11.	Test for Proteins												
	Biuret Test	-	-	-	-	-	-	-	-	-	-	-	-
	Xanthoprotic Test	+	+	+	+	+	+	+	+	+	+	-	+
	Conc.H_2SO_4 Test	-	-	-	+	+	-	+	+	+	+	-	-
12.	Test for Amino acids												
	Ninhydrin Test	-	-	-	- -	-	- -	-	-	-	-		
13.	Test for Terpenoids												
	Salkowshi's Test	++	++	++	+	-	+	++	++	++	++	-	-

Table 2. Antifungal Activity

S. No	Samples Fungal Strains	Acetone stem	Methanol stem	Acetone leaf	Methanol leaf
1	*Aspergillus fumigatus*	20.83 ±0.89	15.66 ± 0.51	7 ± 0	11.5 ± 0.54
2	*Aspergillus niger*	24.33± 0.81	26.33 ± 0.51	12 ± 1.67	13.33 ±2.10
3	*Mucor indicus*	19.16±1.16	11.16 ± 0.75	10.1 ± 0.75	15.5 ± 0.54

Figure 1

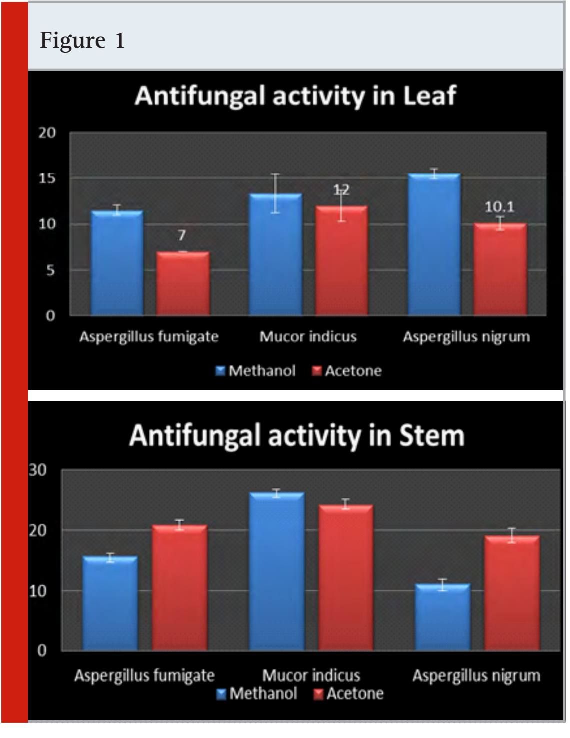

Plate 1

Potato – 200g, Dextrose – 20g,Agar – 20g, Distilled water – 1000ml

pH – 7.0 The PDA was weighed as per the requirement and was dissolved in 1000 ml of distilled water. The pH was adjusted to 7.0 with a digital pH meter. The medium was kept for boiling until complete dissolution of the ingredients. Then, it was autoclaved for at 151bs pressure and 121°C.Procedure: 20ml PDA containing Petri plates were seeded with the matured culture of fungal strains. Wells were cut using a sterile Cork Borer and 100µl (200µg/well) of extracts were added into the well. For the negative control, distilled water was added to the wells. Then plates were kept for incubation for about a week at room temperature. The antifungal activity was examined by measuring the diameter of the inhibition zone formed around the well in millimeters(mm).

RESULTS AND DISCUSSION

Serving as a phytomedicine, generally plants have contributed to human health and well-being. Firsthand information recorded by ancient physicians were evaluated and detail about the properties and therapeutic values are being investigated in recent times (Shrestha and Dhillion, 2003, Afolayan 2013 and Ganesh et al 2019). The results of qualitative screening of phytochemicals of *H. macrocarpus* leaf and stem

showed the presence of Alkaloids, Carbohydrates, Glycosides, Flavonoids, Phenols, Tannins, and Fixed oils, Sponins, Sterols and Terpenoids. High concentrations of phytochemicals were found in methanolic, ethanolic, acetone and aqueous extracts while a very low concentration in chloroform and petroleum ether extracts (Table 1).

Antifungal Activity: Acetone extracts of leaf and stem were tested for in vitro the antifungal activity against *Aspergillus fumigatus*, *Aspergillus niger* and *Mucor indicus* were investigated. Acetone leaf extract showed highest activity for Aspergillus niger (12 ± 1.67). On the other hand the stem extract showed the highest activity for acetone against the pathogen *Aspergillus niger* (24.33± 0.81). it is seen in the Table 2, figure. Methanol extracts of leaf and stem were tested for in vitro antifungal activity against *Aspergillus fumigatus*, *Aspergillus niger* and *Mucor indicus* were investigated. methanolic leaf extract showed highest activity for Mucor (15.5 ± 0.54). On the other hand the stem extract showed the highest activity for methanol against the pathogen *Aspergillus niger* (26.33 ± 0.51) figure, Plate.

CONCLUSION

The study on the leaf of *H. macrocarpa* for its phytochemical constituents has revealed the presence of secondary metabolites. Methanol, ethanol, acetone and aqueous are good extractive solvents and the antifungal studies have shown the highest inhibition activity against the pathogens *Aspergillus nigrum* and *Mucor indicus*.

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Antibacterial Activity of Pomegranate (*Punica granatum*) Fruit Peel Extracts Against Antibiotic Resistant Gram- Negative Pathogenic Bacteria

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ABSTRACT

Fighting bacterial antibiotic resistance is a great challenge, and the researchers are in search of alternative therapies, the effective antibacterial biotherapeutics, in particular. This research aims to explore the antibacterial potentiality of pomegranate (*Punica granatum*) fruit peel extracts against gram-negative pathogenic bacteria having high MAR (multiple antibiotic resistance) indices. A total of 17 gram-negative pathogenic bacteria: *Escherichia coli* (n=5), *Proteus spp*. (n=4), *Klebsiella pneumoniae* (n=2), *Pseudomonas aeruginosa* (n=3), *Acinetobacter baumannii* (n=3), were subjected to susceptibility testing by disc diffusion method using 15 antibiotics, and the MAR indices were calculated. The antibacterial activities of APE (pomegranate fruit peel aqueous extract) and PEE (pomegranate fruit peel ethanolic extract), for the test bacteria, were determined by disc diffusion, while agar dilution technique was followed to determine the MIC (minimum inhibitory concentration) values of the extracts. The bacteria tested, displaying varied MAR resistance phenotypes, had resistance to 7-14 antibiotics, and the MAR indices for the bacterial isolates ranged 0.46–0.93. The PEE and APE both showed antibacterial activities, with respective ZDI (zone diameter of the inhibition) values of 14.7±5.32 mm and 17.53±5.72 mm (at 1 mg/disc), and 13.3±5.69 mm and 16.65±7.55 mm (at 2 mg/disc). The PEE and APE MICs ranged 2.5-3.3 mg/ml and 5–20 mg/ml, respectively, for the test bacteria. Thus, fruit peel of pomegranate might be useful in the preparation of antibacterial therapeutic agents, alternative to antibiotics, in order to combat the life-threatening infections of multiple antibiotic resistant gram-negative bacteria.

KEY WORDS: *POMEGRANATE FRUIT PEEL, ANTIBACTERIAL ACTIVITY, MINIMUM INHIBITORY CONCENTRATION, MAR INDICES, GRAM-NEGATIVE PATHOGENIC BACTERIA.*

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INTRODUCTION

The medicinal and food plants have been in use, for centuries, in treating infectious diseases, and have been considered as important source of antimicrobial agents, and for decades, their (plants) antimicrobial properties have been investigated in curing a variety of bacterial infections, and combating bacterial antibiotic resistances, as well (Alanis et al. 2005; Nozohour et al.2018, Matjuda and Aiyegoro (2019). The *Punica granatum* (pomegranate; family: Punicaceae; Bedana in Bengali) fruit peel is an important inedible part, possessing an enormous amount of flavonoids, tannins and other phenolic compounds (Khan et al. 2017; Janani et al. 2019) and thus displaying various kinds of bioactivities including antioxidative and antimicrobial properties.

(Devatkal et al. 2013; Voravuthikunchai et al. 2005; Reddy et al. 2007). Devatkal et al. (2013) reported the antibacterial activity of aqueous extract of pomegranate peel against poultry meat isolates of *Pseudomonas stutzeri*. Navidinia and Goudarzi (2017) demonstrated the MICs of aqueous and ethanolic extracts of *P. granatum* seeds that ranged 9.37- 150 mg/ml and 9.37- 75 mg/ml, respectively, for various gram-negative potential bacterial pathogens. The pomegranate edible and non-edible parts have been reported to be excellent antibacterial as well as antioxidative agents containing rich amount of polyphenolics (Rummun et al. 2013).The pomegranate fruit parts: peel, aril, seeds, and juice, have been reported to be rich in different bioactive components, as has been demonstrated by Jurenka et al. (2008).

The gram-negative bacteria, such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii*, are top listed WHO (World Health Organization) priority pathogens, and some of the members are included in the ESKAPE (*Enterococcus faecium,*

Table 1. Multiple antibiotic resistance (MAR) phenotypes and MAR indices for clinical bacterial isolates (n=17)

Bacteria	Resistance	MAR phenotypes	MAR index
A. baumannii AB1	7-drug	Vm-Am-Mc-Km-Tr-Cpd-Nx	0.46
E. coli EC3	8-drug	Cx-Vm-Am-Ip-Mc-Tr-Cpd-Nx	0.53
E. coli EC4	9-drug	Cx-Cf-Vm-Am-Ip-Mc-Tr-Cpd-Nx	0.6
P. vulgaris PV2	9-drug	Cf-Cp-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.6
E. coli EC2	10-drug	Cx-Vm-Tc-Cp-Am-Ip-Mc-Tr-Cpd-Nx	0.66
A. baumannii AB2	11-drug	Cm-Cx-Cf-Vm-Am-Mc-Ak-Km-Tr-Cpd-Nx	0.73
K. pneumoniae KP2	11-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Km-Cpd	0.73
A. baumannii AB3	12-drug	Gm-Cm-Cx-Cf-Tc-Am-Ip-Mc-Ak-Km-Tr-Cpd	0.8
K. pneumoniae KP1	12-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Tr-Cpd-Nx	0.8
P. aeruginosa PA3	12-drug	Cm-Cx-Cf-Vm-Tc-Cp-Am-Mc-Km-Tr-Cpd-Nx	0.8
P. mirabilis PM1	12-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.8
E. coli EC5	13-drug	Cm-Cx-Cf-Vm-Tc-Cp-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.86
P. mirabilis PM2	13-drug	Gm-Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Km-Tr-Cpd-Nx	0.86
P. vulgaris PV1	13-drug	Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Km-Tr-Cpd-Nx	0.86
E. coli EC1	14-drug	Gm-Cx-Cf-Vm-Tc-Cp-Am-Ip-Mc-Ak-Km-Tr-Cpd-Nx	0.93
P. aeruginosa PA1	14-drug	Gm-Cm-Cx-Cf-Vm-Tc-Am-Ip-Mc-Ak-Km-Tr-Cpd-Nx	0.93
P. aeruginosa PA2	14-drug	Gm-Cm-Cx-Cf-Vm-Tc-Cp-Am-Mc-Ak-Km-Tr-Cpd-Nx	0.93

Ak: amikacin; Am: ampicillin; Cf: cefotaxime; Cx: cefoxitin; Cpd: ceftazidime; Cp: ciprofloxacin; Cm: chloramphenicol; Gm: gentamycin; Ip: Imipenem; Km: kanamycin; Mc: methicillin; Nx: nalidixic acid; Tc: tetracycline; Tr: trimethoprim; Vm: vancomycin

Table 2. The ZDI (zone diameter of inhibition) values of pomegranate fruit peel extracts for clinical bacterial isolates (n=17)

Bacteria	ZDI (mm)			
	PEE (1 mg/disc)	APE (1 mg/disc)	PEE (2 mg/disc)	APE (2 mg/disc)
E. coli EC1	10	14	13	22
E. coli EC2	13	15	14	23
E. coli EC3	10	8	12	11
E. coli EC4	13	18	15	26
E. coli EC5	22	20	25	22
A. baumannii AB1	22	20	28	25
A. baumannii AB2	14	6	18	6
A. baumannii AB3	10	6	12	6
P. mirabilis PM1	19	16	22	20
P. mirabilis PM2	15	15	20	18
P. vulgaris PV1	22	24	26	27
P. vulgaris PV2	25	18	26	24
K. pneumoniae KP1	10	6	10	6
K. pneumoniae KP2	8	8	13	11
P. aeruginosa PA1	10	6	12	6
P. aeruginosa PA2	12	10	14	12
P. aeruginosa PA3	15	16	18	18
Mean	14.7	17.53	13.3	16.65
SD	5.32	5.72	5.69	7.55
p value	0.47		0.71	

The abbreviation of the plant extracts are mentioned in the text

Staphylococcus aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and Enterobacter spp.) group (Rice, 2008; Smith et al. 2018; Perovic et al. 2018). For such bacterial pathogens, the gram-negative bacteria, in particular, having the capacity to cause severe nosocomial infections (and non-responsive to currently available antibiotics), newly developed effective therapies are required (Tacconelli et al. 2018). Both edible and non-edible parts of pomegranate plant have been reported to treat different pathological conditions in different traditional medicine (Derakhshan et al. 2018). Therefore, the current study was undertaken to authenticate the antibacterial capacity *P. granatum* fruit peel (available in the local niches: West Bengal, India) against *E. coli, A. baumannii, P. aeruginosa, K. pneumoniae*, and *Proteus spp.* (*P. mirabilis* and *P. vulgaris*) showing resistance to multiple antibiotics.

MATERIAL AND METHODS

Bacterial Strain and Media: A total of 17 clinical bacterial isolates: *Escherichia coli* (n=5), *Proteus spp.* (n=4), *K. pneumoniae* (n=2), *P. aeruginosa* (n=3), *A. baumannii* (n=3), which were maintained in the laboratory in cystine tryptone agar stabs, were utilized in the current study. The media (Hi-Media, India) used in the study were nutrient broth (for bacterial subculture and inoculums preparation) and nutrient agar (for antibiotic susceptibility and antibacterial activity testing).

Antibiotic Susceptibility: The antibiotic susceptibility testing, for the bacterial isolates, was done following disc diffusion (Bauer et al., 1966), using 15 antibiotics (Hi-Media, India): ampicillin (Am; 10-µg), amikacin (Ak; 30-µg), cefoxitin (Cx; 30-µg), cefotaxime (Cf; 30-µg), cefpodoxime (Cpd; 10-µg), chloramphenicol (Cm;

30-µg), ciprofloxacin (Cp; 10-µg), gentamycin (Gm; 30-µg), imipenem (Ip; 10-µg), kanamycin (Km; 30-µg), methicillin (Mc; 5-µg), nalidixic acid (Nx; 30-µg), tetracycline (Tc; 30-µg), trimethoprim (Tr; 5-µg), and vancomycin (Vm; 30-µg). The ZDI (zone diameter of inhibition) values from the antibiotic action against the test bacteria were recorded, and interpreted according to the CLSI protocol (CLSI, 2015). The MAR indices for the bacteria tested were calculated following the formula as stated by Nandi and Mandal (2016), and the results were interpreted according to the criteria published earlier (Krumperman, 1983). The MAR phenotypic profiles were determined for the bacterial isolates displaying resistance to three or more antibiotics (Adefisoye and Okoh, 2017).

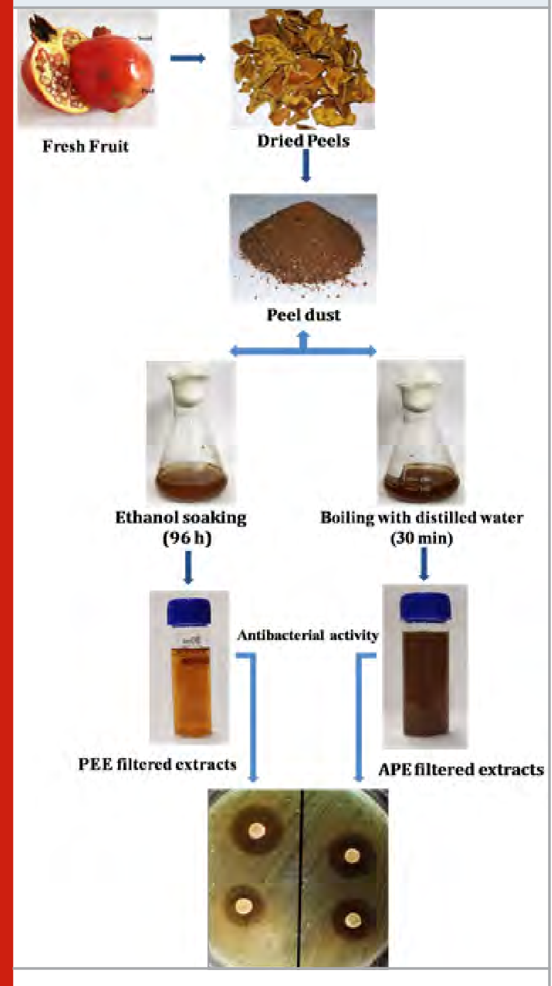
Plant Extract Preparation: The indigenous variety fruits of pomegranate, *Punica granatum* (family: Punicaceae) were collected from Rajapur village of Malda district (West Bengal, India), washed properly with distilled water, and the peels were separated and sliced for shade drying. The dried plant materials were granulated by electrical grinding machine and stored in airtight containers at room temperature for extract preparation. The pomegranate fruit peel ethanolic extract (PEE) and pomegranate fruit peel aqueous extract (APE), were prepared in line with a little modification of the protocol depicted by Sircar and Mandal (2016). Briefly, for PEE preparation, 5 g of dried pomegranate fruit peel granules was extracted

Table 3. The MIC (minimum inhibitory concentration) values of pomegranate fruit peel extracts for clinical bacterial isolates (n=17)

Bacteria	MIC (mg/ml)	
	PEE	APE
E. coli EC1	2.5	5
E. coli EC2	3.3	5
E. coli EC3	3.3	5
E. coli EC4	3.3	5
E. coli EC5	3.3	20
A. baumannii AB1	2.5	6.6
A. baumannii AB2	2.5	6.66
A. baumannii AB3	2.5	16.66
P. mirabilis PM1	2.5	6.6
P. mirabilis PM2	2.5	6.6
P. vulgaris PV1	2.5	6.6
P. vulgaris PV2	2.5	6.6
K. pneumoniae KP1	3.3	20
K. pneumoniae KP2	3.3	20
P. aeruginosa PA1	2.5	11.6
P. aeruginosa PA2	2.5	6.6
P. aeruginosa PA3	3.3	13.33
Mean	2.83	9.65
SD	0.41	±5.8
p value	0.00012	

The abbreviation of the plant extracts are mentioned in the text":

Figure 1. Flow diagram for antibacterial activity analysis of pomegranate fruit peel ethanolic and aqueous extracts



The abbreviation of the plant extracts are mentioned in the text":

by soaking with 100 ml of ethanol shaking at regular interval, for 96 h at room temperature, and sieved through cheese-cloth and Whatman No. 1 filter paper. For the preparation of APE, 5 gm granulated sample was dissolved in 100 ml of double distilled water, and boiled for 30 min in water bath, and filtered as mentioned above, after cooling. The concentration of each of the extracts (APE and PEE) in stock solution was 50-µg/µl. The extracts prepared were stored at 4°C until further used.

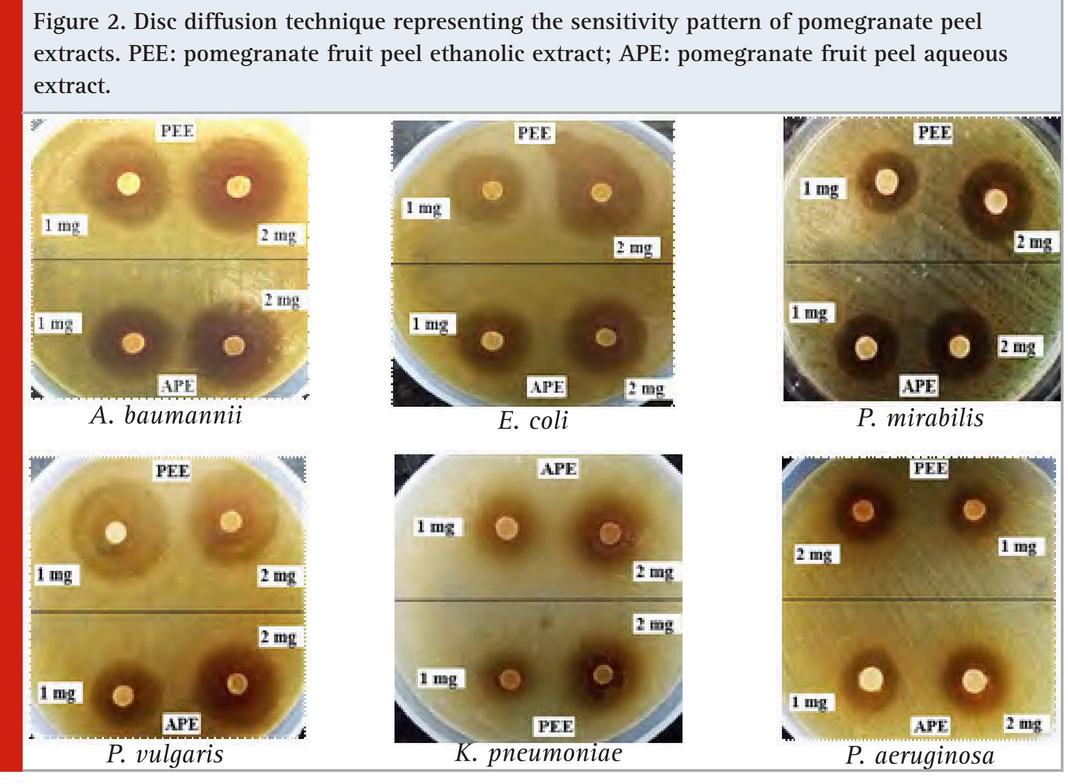
Antibacterial Property: The antibacterial activity of PEE and APE extracts were evaluated employing disc diffusion technique (in order to get the zone diameter of the inhibition; ZDI), as explained earlier by Das and Mandal (2016). Agar dilution method, the details of which was mentioned in previous publication (Mandal et al. 2007), was followed for the determination of MIC (minimum inhibitory concentration) values, using nutrient agar medium mixed with varied concentration of the extracts, ranging from 2.5 to 3.3 mg/ml and 5 to 20 mg/ml. The all incubations were done at 37°C for 24 h, and the testing was at once completed in triplicate. The antibacterial activity was recorded based on the ZDIs obtained around the plant extract impregnated discs on the agar plates inoculated with test bacteria, and the ZDI values ≥7 mm accounted sensitivity of the test extracts to the bacterial isolates (Nascimento et al. 2000). The lowest extract concentration that inhibited the visible growth of the test bacteria were defined as MICs (Mandal et al. 2007).

Statistical Analysis: To compare the antibacterial activity (in terms of ZDIs) by disc diffusion technique, and MICs of plant extracts: APE and PRE, against the gram-negative pathogenic bacteria tested, the data were expressed as the mean ± SD (standard deviation), and were evaluated by 't'-test , using MS Excel 2010 software; the statistical significance was projected by 'p' value of ≤ 0.05.

RESULTS AND DISCUSSION

The current research explores the antibacterial activity of pomegranate fruit peel ethanolic and aqueous extracts against antibiotic resistant

Figure 2. Disc diffusion technique representing the sensitivity pattern of pomegranate peel extracts. PEE: pomegranate fruit peel ethanolic extract; APE: pomegranate fruit peel aqueous extract.



gram-negative pathogenic bacteria (Figure 1). The multiple antibiotic resistance phenotypes for the test bacterial pathogens are represented in Table 1; the isolates showed 7-drug to 14-drug resistances, displaying the respective resistance patterns: 'Vm-Am-Mc-Km-Tr-Cz-Nx', for *A. baumannii* AB1 strain, and 'Gm-Cm-Cx-Cf-Vm-Tc-Cp-Am-Mc-Ak-Km-Tr-Cz-Nx' for *E. coli* EC1, *P. aeruginosa* PA1 and *P. aeruginosa* PA2 strains. As per the report of Matjuda and Aiyegoro (2019), among a total of 74 resultant MAR phenotypes (ranging from 3-drug to 12-drug resistances), the predominant patterns noted included "penicillin-sulphamethaxazole-Vm-Am-amoxicillin-apramycin-neomycin-tilmicosin-oxytetracycline-spectinomycin-linomycin-Tr" and "penicillin-sulphamethaxazole-Vm-amoxicillin-neomycin-tilmicosin-oxytetracyclin-spectinomycin-linomycin", for 15 and 6 test bacterial isolates, respectively. The MAR index for the test clinical bacteria ranged from 0.46 to 0.93 (Table 1). As has been reported earlier by Matjuda and Aiyegoro (2019), the MAR indices of pathogenic bacteria tested ranged from 0.2 to 1. Das et al., (2018).

Reported different MAR resistance phenotypes, among gram-negative clinical bacteria, which ranged up to 10-drug resistance, displaying the pattern: 'Am-Ce-Cp-Ct-Cx-Mp-Nx-Pc-PT-Tc' by E. coli CSD2 strain and in that study the MAR indices for the test bacteria ranged 0.15 – 0.77. The earlier authors (Tambekar et al. 2005; Kaneene et al. 2007) explained that the bacteria demonstrating MAR indices >0.4 might be originated from niches with human-faecal contamination, while the bacteria displaying MAR indices >0.2, have been regarded to be derived from niches with high antibiotic pollution (Krumperman, 1983; Matjuda and Aiyegoro, 2019). The high MAR indices (0.46 – 0.93) among the test gram-negative clinical bacteria demonstrated, in the current study, their origin from human-faecal contaminated niches with high antibiotic pollution.The antibacterial activity of pomegranate peel extracts against gram-negative pathogenic bacteria, following disc diffusion method, is shown in Figure 2. The ZDIs from the action of APE and PEE, against the test bacteria, are represented in Table 2. The PEE and APE had ZDIs 10–22 mm and 8–20 mm, respectively (at 1.0 mg/well), and 12–25 mm and 11–26 mm, respectively (at 2.0 mg/well), against *E. coli* isolates. The PEE was active against all the test *A. baumannii* isolates (ZDIs 10 – 28 mm), while the APE showed activity against *A. baumannii* AB1 isolate only. The pomegranate fruit peel showed anti-Proteus spp. activity with ZDIs 15 – 26 mm, for PEE, and 15 – 27 mm, for APE. The PEE had growth inhibitory activity against all the *K. pneumoniae* isolated tested (n=3; ZDIs: 8 – 14 mm). The pomegranate peel extract had ZDIs of 10 – 18 mm against *P. aeruginosa*; however, for *P. aeruginosa* PA1 the APE had no activity (ZDI: 6 mm). As per the report of Algurairy (2018) the pomegranate fruit peel ethanolic extract (10 – 100 %) had ZDIs of 22–36 mm, for Staphylococcus aureus clinical isolates. The respective ZDIs of pomegranate fruit peel methanolic and aqueous extracts (50 mg/ml) for *Enterobacter cloacae* were 14 mm and 10 mm, and for *Salmonella enterica* serovar Typhi, 20 mm and 10 5mm, while for the gram-positive (*S. aureus* and *Bacillus subtilis*) bacteria, the ZDIs ranged 22–26 mm and 24–28 mm, respectively (Kanoun et al. 2014).

The methanolic extract of pomegranate fruit peel showed antibacterial activity against food-borne bacteria, such as, *Listeria monocytogenes, S. aureus, E. coli* and *Yersinia enterocolitica* (Al-Zoreky, 2009). As has been reported by Kunte et al. (2018), the pomegranate fruit peel aqueous extract had antibacterial activity against potential cariogenic *Streptococcus mutans* isolates, displaying ZDIs of 15 – 17 mm. The pomegranate peel fresh aqueous extract showed growth inhibitory activity against *Pseudomonas stutzeri* isolates from poultry meat displaying ZDIs of 21 – 26 mm (Devatkal et al. 2013). The pomegranate peel extract showed antibacterial activity against S. mutans and *Streptococcus mitis* having ZDIs of 20 mm and 25 mm, respectively, while the leaf extract had ZDIs of 16 mm and 18 mm, respectively, for the bacterial isolates (Rummun et al. 2013). The *P. granatum* seed ethanolic extract showed antibacterial activity against gram-positive bacteria: *S. aureus*, with ZDIs 22–42 mm as well as gram-negative bacteria: *E. coli*, having ZDIs 27–42 mm, while the respective ZDIs of petroleum ether extract for the isolates ranged

16–34 mm and 15–27 mm (Bora et al. 2018). The two extracts, APE and PEE, by disc diffusion (Table 2), displayed growth inhibition activities against the test bacteria, wherein there was no significant difference between the antibacterial properties of APE and PEE (p values: 0.47 – 0.71). The MICs of PEE ranged 2.5-3.3 mg/ml for all the gram-negative pathogenic bacteria tested, while the APE MICs ranged from 5 mg/ml (for *E. coli* EC1, *E. coli* EC2, *E. coli* EC3 and *E. coli* EC4) to 20 mg/ml (for *E. coli* EC5), and the APE MICs for the remaining bacterial pathogens ranged 6.6 – 16.66 mg/ml (Table 3). The earlier authors also have demonstrated antibacterial activity of pomegranate extracts against clinically relevant bacteria, from different parts of the globe. The pomegranate peel methanolic and aqueous extract had MICs 0.39 and 0.195 for S. aureus, 1.56 and 0.78 mg/ml, for *B. subtilis*, 3.125 and 1.56 mg/ml, for *E. cloacae*, and 12.5 and 6.25 for S. enterica serovar Typhi (Kanoun et al. 2014). The respective MICs of aqueous and ethanolic pomegranate seed extracts for the bacteria tested were: *E. coli* (75 and 37.5 mg/ml), *Shigella sonnei* (37.5 and 18.75 mg/ml), *Shigella flexneri* (18.75 and 9.37 mg/ml), *Shigella dysentery* (18.75 and 9.37 mg/ml), *P. vulgaris* (18.75 and 9.37 mg/ml), *P. mirabilis* (9.37 and 9.37 mg/ml) and *Citrobacter fraundii* (150 and 75 mg/ml), as demonstrated by Navidinia and Goudarzi (2017).

Prashanth et al. (2001) reported antibacterial activity of pomegranate fruit extract against grem-negative bacteria: *E. coli*, *K. pneumoniae*, *P. vulgaris* and *S. enterica* serovar Typhi, in terms of MICs, which were 12 mg/ml, 12-25 mg/ml, 1.5-12 mg/ml, 12 – 50 mg/ml, for petroleum ether extract, chloroform extract, methanol extract and aqueous extract. The pomegranate fruit methanol and aqueous extracts had MICs, against gram-negative bacterial strains: *E. coli* ATCC 25922, *S. dysantriae* PTCC 1188 and *S. enterica* serovar Typhi ATCC 19430, of 6.25-12.5 and 3.12-12.5 mg/ml, respectively, as has been reported by Mahboubi et al. (2015). On the basis of the MIC determination (Table 3), the pomegranate fruit peel extracts, APE and PEE, were well active against the gram-negative multiple antibiotic resistant pathogenic bacteria; the APE, however, displayed greater activity compared to the PEE (p value: 0.00012).

CONCLUSION

The fruit peel ethanolic as well as aqueous extracts of pomegranate displayed antibacterial activity against gram-negative bacteria having high multiple antibiotic resistance indices, suggesting the usefulness of the of the plant parts in the preparation of antibacterial bio-therapeutics that might be utilized in the treatment of diseases caused due to the infection of multiple antibiotic resistant gram-negative bacteria. Further, phytochemical analysis and pharmacokinetic studies are required to explore the bioactive components responsible for antibacterial activity, and to determine the effective dosage

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Modified Direct Differential Coding Using 2D-Dynamic Dictionary for Nucleotide Sequence

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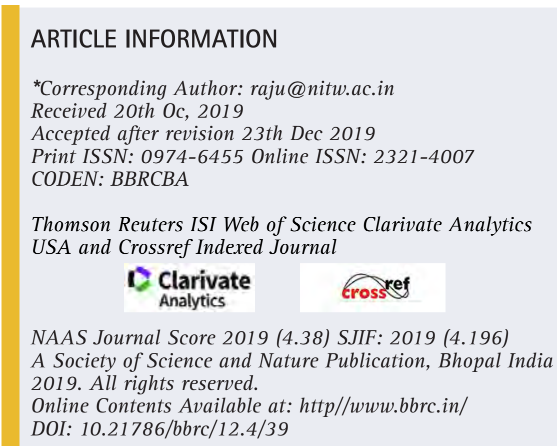
ABSTRACT

Bioinformatics is the application of computer technology for the management of biological information. As the exponential growth of DNA sequence is very big in size, ranging from 3 to 50 GB or more depending on the species. Being large in size, molecular data imposes restrictions in searching and retrieval of sequences. While modern hardware provides vast amounts of inexpensive storage. The compression of biological sequence data is still important in order to facilitate fast search and retrieval operations by reducing the original size of the sequence. The Differential Direct Coding technique is DNA sequence data compression algorithm that identifies sequence data and auxiliary data by including the extra codes for the symbols that are other than the set of nucleotide bases. The Differential Direct Coding algorithm is a single phase algorithm which removes the overhead of handling wildcard symbols. Here the algorithm works on construction dictionary based coding technique that provides better compression ratio in spite of little storage space with the reference of Differential Direct Coding algorithm.

KEY WORDS: AUXILIARY DATA, BIOINFORMATICS, COMPRESSION RATIO, DIFFERENTIAL DIRECT CODING.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INTRODUCTION

Bioinformatics is a multidisciplinary science that uses methods, principles from mathematics, computer science for analyzing the biological data. The computer databases and algorithms are developed to speed up and enhance biological research. A fundamental requirement for research in bioinformatics is the capacity to warehouse large amounts of biological sequence data (Bekas, Konstantinos.,2019). Genomic repositories contain a large amount of data, due to this some efficient algorithms have emerged to facilitate communication and storage issues. Out of this, cheap storage is not a big issue as due to limited bandwidth communication of modern world (Salomon and Motta 2010). The amount of DNA sequenced from organisms is increasing rapidly (Cao et al 2007). Sequencing initiatives are contributing exponentially increasing quantities of nucleotide data to databases such as GenBank (Williams, et.al., 1997, Benson, et.al., 2008).

As of January 2009, the Nucleic Acids Research online Molecular Biology Database Collection listed 1170 publicly available biological databases(Galperin and Cochrane, 2008). GenBank, a major sequence database and a component of the International Nucleotide Sequence Databases (INSD), doubles in size roughly et al. 2008). In January 2013 the real cost of sequencing human-size genome (according to NHGRI data) was about 5,700 dollars, while the cost of one-year storage at Amazon S3 and 15 downloads of raw reads and mapping results (225 GB of reads with 30-fold coverage and 500 GB of mapped data) was close to 1,500 dollar (Deorowicz and Grabowski 2013).

Compression of biological sequences is useful, not primarily for managing the genome database, but for modeling and learning about sequences (Gauthier, Jeff, et al., 2018). Since DNA is the "instruction of life", it is expected that DNA sequences are not random and can be compressible. While some DNA sequences are highly repetitive. A repeat subsequence is a copy of a previous subsequence in the genome, either forward or reverse complement. Most DNA repeats are not exact as nucleotides can be changed, inserted or deleted. It is estimated that 55% of the human genome is repeat DNA. Because of the particularity of the DNA sequence data, it is not very ideal with traditional compression algorithms, (Cao et al 2007). There are many methods to achieve the compression of the data, (Das et.al, 2005, Chen et.al. 2001,Galperin et.al., 2008 Jifeng, et.al. 2012, Cao et.al., 2007). This research particularly concentrate on the table method(dictionary method).

There exist many algorithms based on dictionary method like Ziv-Lampel (Vey, 2009, Das et al 2005). Also some other arithmetic encoding algorithms also like Huffman algorithm. However, these universal text compression algorithms are not suitable for compression of biological sequences as they consider the sequence as a pure text stream. Nucleotide symbols deal with four symbols representing nucleotide bases {A, C, T, G}. Earlier, many text compression techniques have been applied on biological data, which are less efficient. Interestingly, most general purpose text compression algorithms fail to compress DNA to below the naive 2 bits per symbol. That is because DNA regularities are different from those in text and are rarely

Table 1. Differential Direct Coding (2D) with Dictionary Model

Type of Data	Description	Range	Dictionary Part
Auxiliary Symbol (Wildcards)	ASCII	0 to 127	
Triplets	Set of three base characters	-1 to -64	Fixed Part
Multiple of Triplets	Set of 3,6,9,12, base characters	-65 to -127	Dynamic Part

modeled by those compressors(Cao et al 2007). Thus there appeared compression algorithms for DNA sequence specially. A number of special purpose compression algorithms for DNA have been developed recently. The algorithms can be divided into two kinds: based on substitution and based on statistic (Jifeng 2012). The former class replace a long repeated subsequence by a pointer to an earlier instance of the subsequence or to an entry in a dictionary. Examples of this category are the popular Lempel-Ziv compression algorithms and their variants (Laird, Sarah, and Rachel Wynberg 2018)

On the other hand, a statistical compression encoder such as prediction by partial match (PPM) (Lipmann et al. 2008) predicts the probability distribution of each symbol(Cao et al 2007). The Huffman coding (Salomon et al 1952) invented in 1952, is a statistical method, which assigns a sequence of bits (a codeword) to each alphabet symbol. The code words are of different length, and in accordance to the golden rule of data compression, rarer symbols are represented by longer code words. The given sequence is then encoded by replacing each symbol with its corresponding codeword. Huffman coding importance is its optimality, i.e., no other code leads to a shorter encoded sequence. In 1977–78 Ziv and Lempel (Salomon Motta, 2010, Ziv and Lempel 1977) invented dictionary methods. They have processed the sequence from left to right and encode possibly long repetitions of consecutive symbols as references to the already compressed part of data. Such an approach allows for higher compression ratios than Huffman coding as it looks for another type of redundancy. Not only in natural language (e.g., repeating word phrases), but also in multiple genome sequences or overlapping sequencing reads. Even better results are possible with combining dictionary methods and Huffman coding. While modern hardware can provide vast amounts of inexpensive storage, the compression of biological sequence data is still of paramount concern in order to facilitate fast search and retrieval operations, primarily by reducing the number of required I/O operations, (Galperin and Cochrane, 2008). Data compression requires two fundamental processes: modelling and coding (Williams and Zobel, 1996).

Table 2. Dictionary Table

Triplet	ASCII Value
AAA	1
AAC	2
-----	---
TTT	64
AAAAAC	65
AACCGT	66
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AACCGTGTA	127

Modeling involves constructing a representation of the distinct symbols in the data, along with any associated data, like the relative frequencies of the symbols (Williams and Zobel, 1996). Coding involves applying the model to each symbol in the data to produce a compressed representation of the data, preferably by assigning short codes for frequently occurring symbols and long codes to infrequently occurring symbols (El Naqa et al. 2018). In the case of DNA sequences, the finite set of nucleotide symbols {A, C, G, T} can be efficiently modeled as a corresponding set of binary values {00, 01, 10, 11}(Williams and Zobel, 1996). This model constitutes an effective binary representation where each nucleotide base is directly coded by two bits. This assumes that sequence data are indeed composed solely from the four symbols of the nucleotide set. However, this assumption is not guaranteed to be met and a nucleotide sequence may include additional wildcard symbols, like N or S (Williams and Zobel 1996).

Therefore, to reconcile the potential occurrence of symbols other than the expected four nucleotide bases, any unexpected symbol is randomly converted into one of the valid symbols that it represents (Williams and Zobel 1996). Eliminated wildcards are subsequently restored during sequence decompression (Williams and Zobel 1996). This makes the process as 2 phase process. This paper presents substitution based modified direct differential coding algorithm with dynamic dictionary. It is a nucleotide sequence data compression algorithm based on substitution technique with the help of dynamic dictionary.

This algorithm considers triplets and sequences of length multiple of three and it is stored in fixed part and dynamic part of the dictionary. This algorithm is also capable to handle symbols other than four nucleotide bases. It provides better performance as compared to existing algorithm. The Differential Direct Coding is designed by considering fixed size dictionary i.e. it contains 64 dictionary entries for possible combinations of A, C, G and T with set of 3 characters and multiples of it. We have modified the dictionary to contain more than 64 entries where first 64 entries are for triplets and next entries are for sequences of length multiple of 3.

MATERIALS AND METHODS

Initially the method identifies repeat regions multiple of three in the individual sequence and the repeat regions are store in the dictionary table. The proposed algorithm compresses both repeat and non repeat sequences. It also handles the non base character and compresses any nucleotide sequence. If the case of formation of triplets is considered, with combination of four symbols {A, C, G, T} or {A, C, G, U} for DNA or mRNA respectively, then it encounter maximum 64 combinations. These 64 triplet combinations handled by 64 non-printable ASCII characters, whereas there exist total 127 non-printable ASCII characters. Therefore, the remaining 63 characters are used to store some other combinations of size more than 3, which can yield a better compression.

Model: We consider the ASCII characters between the ranges 0 to 127 for the Auxiliary symbols. The ASCII values from -1 to -64 are used for triplets of {A, C, G, and T} or {A, C, G, and U}. The ASCII values from -65 to -127 are used for the sequences which are multiples of 3. The first 64 entries of dictionary are fixed length entries, i.e. sequence of length 3. The entries from 65 to 127 are dynamic length entries, i.e. sequences of length multiple of 3 as shown in Table1. The sample dictionary view is shown in Table. 2.2. It contains 127 entries for different triplets and patterns of length multiple of three. The first 64 entries are for triplets only and remaining upto 127 are for patterns of length multiple of three.

Compression procedure: Apply the model described above, for the encoding of input sequence. While scanning the input sequence the algorithm always searches for the triplet from the fixed size Dictionary Table. It will further scan next triplet therefore the total scanned characters will be of length 6 now. If this combination of 6 characters is available in the dynamic part dictionary table, it will scan next triplet, otherwise it will write the ASCII character corresponding to the last match group in the output file and also insert the current combination into the dynamic part of dictionary table.

This process will be carried on till the whole sequence is encoded. Once the dictionary entries are filled completely, i.e. all 127 entries are filled; the algorithm will not add any more entries in the dictionary and just search for the combinations from both parts of the table. Also this will be followed by writing the ASCII value corresponding to that combination to the output file. The output file is the required compressed file, when the whole sequence is scanned. While scanning the input sequence, in some cases may occur where a triplet cannot be formed. Here the algorithm writes single character or a group of two into the output file as it is. Whenever a wildcard symbol is obtained, the algorithm stops scanning the triplets and writes wildcard character's ASCII encoded equivalent, followed by its positive integral repeats in its decimal form.

Algorithm: Differential Direct Coding (2D) Compression with Dynamic Dictionary Approach

Input: DNA Sequence data file.
Output: Compressed DNA Sequence data file.
Note: Fixed part of dictionary table is preprocessed.

Initially T=NULL, TP=NULL,SEQ=NULL;
Step 1: Read first triplet (T).
IF (T!= NULL)
go to step 2.
ELSE IF (!triplet)
Go to step 5.
END IF
Step 2: Check that T has all nucleotide symbols.

IF (true)
goto step 3.
ELSE
IF (T has wildcard Symbol)
go to step 4
ELSE
go to step 5.
END IF
END IF
Step 3: IF (TP exists in the Dictionary Table)
SEQ=TP
ELSE
Output the code (character) for s
Add ST to the Dictionary Table;
SEQ=T;
END IF
Step 4:
a. Search wildcards and get count of successive wildcards.
b. Replace it with wildcard and count
c. Go to step 6.
Step 5: IF (! triplet)
Write remaining symbols
Go to step 6.
END IF
Step 6: Return to step 1 if EOF is not reached.

Decompression procedure: Decoding phase replaces each signed byte from compressed file with corresponding triplet or sequence of length multiple of three. Decoding starts with reading a byte by byte compressed file. Each read byte is checked whether it is a positive signed byte or negative signed byte. Suppose it is positive signed byte, then it is clear that particular byte is representing an uncompressed value which might be any of wildcard symbols. In this case write that particular byte as it is in output file, and read next byte from compressed sequence file. If read byte is representing a negative byte value then it is clear that MSB of the byte is set which is representing compression bit. It means that particular byte is representation for sequence. Check the dictionary entries against read byte and retrieve the sequence against that byte.

Write that sequence to output file and read next byte from compressed file. Whenever read byte is positive byte, it is representation of wildcard symbol and wildcard symbol follows the decimal number representing number of occurrences of that symbol and write these many numbers of symbols in output file.

Algorithm
Differential Direct Coding (2D) Decompression with Dynamic
Dictionary Approach
Input: Compressed DNA File.
Output: Decompressed Sequence File.
Note: Fixed part of dictionary table is preprocessed.
Step1: WHILE (!EOF)
Read byte b from input file and go to step 2.
IF (EOF)
go to step 5.
IF END
Step 2: If (b is positive) // (Representing Wildcard Symbol)
Read next bytes representing digits and store in an array arr
Convert array arr into integer i and goto Step 3
Else,
go to step 3.
END IF
Step 3: FOR(0 to i)
Write wildcard symbol to output file.
END FOR
Go to Step 1
Step4: Search the Dictionary table for a value –(b) and retrieve corresponding triplet t.
Write triplet t in an output file and go to step 1.
END WHILE
Step 5: Stop.
Example of Differential Direct Coding Compression

Input Sequence: TGACGATGACGATTTNNNAG
Step 1: Read first triplet t=TGA and check for presence in the dictionary. It is present. Then read next triplet.

Step 2: Next triplet t=CGA, and check for presence in the dictionary. It is present so add earlier triplet TGA and CGA to form sequence TGACGA. Check for presence in the dictionary. It is not present in dictionary.
Add TGACGA=$ in the dictionary.
Output for earlier pattern i.e. TGA=#.

Step 3: Read next triplet t= TGA and check for presence in the dictionary. It exists, add earlier triplet CGA and TGA to form sequence CGATGA. Check for presence in dictionary. It is not present in dictionary.

Output for earlier pattern i.e. CGA =+. Add CGATGA =@ in the dictionary.

Step 4: Read next triplet t= CGA and check for presence in dictionary. It is present so add earlier triplet TGA and CGA to form sequence TGACGA. Check for presence in dictionary. It is present in the dictionary.

Read next triplet t=TTT and earlier pattern TGACGA and TTT to form TGACGATTT. Check for presence in dictionary. It is not present in the dictionary.

Add TGACGATTT=& in the dictionary.
Output for earlier pattern i.e. TGACGA=$.
Step 5: Next symbol is non-nucleotide (wildcard) symbol so write output for earlier triplet.
Output earlier pattern i.e. TTT=ˆ.

Step 6: Read wildcard symbols until nucleotide is encountered. Put wildcard symbol with count of occurrence.
Output NNN= N3.
Step 7: Read next triplet t=AG. We got less symbols for triplet, so write as it is to output file.
Output = AG.
Compressed Sequence: #+$ˆN3AG

As the input sequence size is of 20 characters in size whereas the compressed file size is of 8 characters. The compression ratio depends upon the type of the sequence.

Example of the Decompression
Compressed Sequence: #+$ˆN3AG
Step 1: Read first byte b=# and check for positive or negative byte value. It is negative, so put corresponding sequence form dictionary to output file.

Output=TGA.
and go to the next step.
Step 2: Read next byte b=+ and check for positive or negative byte value. It is negative, so put corresponding sequence form dictionary to output file.

Table 3. Table of 2D with Dictionary Approach

Input Sequence	Current Triplet	Sequence of length multiple of 3	Dictionary Table		Output Symbol
			Status of Triplet In Dictionary	Corresponding Entry from Dictionary	
TGACGATGAC GATTTNNNAG TGA	TGA	TGA	Found	TGA = #	
	CGA	TGACGA	Not Found	Add with TGACGA = $ And output TGA=#	#
CGA	CGA	CGA	Found	CGA=+	
	TGA	CGATGA	Not Found	Add CGATGA=@ And output CGA=+	#+
TGA	TGA	TGA	Found	TGA=#	
	CGA	TGACGA	Found	TGACGA=$	
	TTT	TGACGATTT	Not Found	Add TGACGATTT=& and output TGACGA=$	#+$
TTT	TTT	TTT	Found	TTT=ˆ (Next is wildcard)	#+$ˆ
NNN					#+$ˆN3
AG		<3		#+$ˆN3AG	

Output=CGA.

Step 3: Read next byte from b=$ and check for positive or negative byte value. It is negative, so put corresponding sequence form dictionary to output file.

Table 4. Experimental Results of 2D with Dynamic Dictionary with the 2D algorithm

Source Genome	Input File Size (bytes)	2D Algorithm				2D with Dynamic Dictionary(proposed)			
		Compre-ssed File Size	Compre-ssion Ratio	Compress-ion Time (ms)	Decomp-ression Time (ms)	Compress-ed File Size	Compre-ssion Ratio Time (ms)	Compress-ion Time (ms)	Decomp-ression Time (ms)
Bacillus Subtilis	4274764	1404994	3.0425	64466	34945	1376213	3.1061	64631	31741
Escherichia Coil K12 MG1655	4705920	1567898	3.0014	70950	38678	1513218	3.1098	70646	37372
Mycoplasm Genitalium G37	588365	193362	3.0428	5362	4683	185424	3.1730	8845	4267

Output=TGACGA.

Step 4: Read next byte from b=ˆ and check for positive or negative byte value. It is negative, so put corresponding sequence form dictionary to output file.

Output=TTT.

Step 5: Read next byte from b=N and check for positive or negative byte value. It is positive and a wildcard symbol, so read next decimal number i.e. 3 and write these many Ns to output file.

Output=NNN.

Step 6: Read next byte b=A and check for positive or negative. It is positive but not a wildcard, so write as it is to output file.

Output=A

Step 7: Read next byte b=A and check for positive or negative. It is positive but not a wildcard, so write as it is to output file.

Output=G

Decompressed Sequence:

TGACGATGACGATTTNNNAG

RESULTS AND DISCUSSION

Here, by referring the Differential Direct Coding (2D) algorithm, we have proposed the algorithm "Differential Direct Coding (2D) based on Dynamic Dictionary Approach", which is working on triplets of nucleotides and patterns of length multiple of three. We have modified the dictionary table of Differential Direct Coding algorithm to divide it in two parts as fix part and dynamic part. The performance of proposed algorithm is compared with base algorithm. The results table contains the original input file size in bytes, compressed file size in bytes by 2D algorithm, the compression ratio for 2D algorithm. It also contains compressed file size in bytes by 2D with Dynamic Dictionary algorithm, the compression ratio for 2D Dynamic Dictionary algorithm. The compression ratio is derived from following formula-

Compression Ratio = Original File Size / Compressed File Size.

It was observed that 2D with Dynamic Dictionary is producing an output file of small size as compared to 2D algorithm. For Bacillus subtilis 2D is generating output file of size 1404994 bytes resulting a compression ratio 3.0425. Whereas 2D with Dynamic Dictionary is generating an output file of size 1376213 which is 28781 bytes less than 2D output file resulting a compression ratio 3.1061. For Escherichia coli K12 MG1655 2D is generating output file of size 1567898 bytes resulting a compression ratio 3.0014. Whereas 2D with Dynamic Dictionary is generating output file of size 1513218 which is 54680 bytes less than 2D output file resulting a compression ratio 3.1098. For Mycoplasm genitalium G37 2D is generating output file of size 193362 bytes resulting a compression ratio 3.0428. Whereas 2D with Dynamic Dictionary is generating output file of size 185424 which is 7938 bytes less than 2D output file resulting a compression ratio 3.1730.

In the similar grounds the decompression ratios of 2D with dynamic dictionary algorithm when compared with 2D algorithm shows a significant decrease in all the three source genome types mentioned i.e., in Bacillus subtilis there is '9.1%' decrease in compression ratio,in Escherichia coli K12 MG1655 nearly '3.5%' decrease and in Mycoplasm genitalium G37 '8.9%' decrease is observed.The results from Table 4.1 clearly indicate better compression results for all three input files. The compression ratio for 2D with Dynamic Dictionary is also better than compression ratio for 2D. High compression ratio also suggests a highly repetitive sequence.

Thus proposed algorithm, "Differential Direct Coding (2D) with Dynamic Dictionary Approach", has high compression ratio to other existing DNA Sequence Compression algorithm, Differential direct coding: a compression algorithm for nucleotide sequence data. The Table 4.1 shows the execution time of proposed algorithm and Differential Direct Coding (2D) algorithm for three different input files. The execution time from table is the average of ten execution times. From Table 4.1 it is clear that the compression time for proposed algorithm is more than Differential Direct Coding algorithm for all input files. Decompression time of proposed algorithm for Bacillus subtilis and Escherichia coli K12 MG1655 is more than Differential Direct Coding algorithm, whereas for Mycoplasm genitalium G37 it is more than Differential Direct Coding algorithm.

CONCLUSION

A new DNA sequence data compression algorithms by dynamic dictionary approach technique have proposed. Here direct coding on triplet basis technique for modeling and encoding purposed. An experimental result gives efficient results compared with existing techniques and reduces the compression as well as the execution time. From these experiments, we conclude that algorithm "differential direct coding with dynamic dictionary approach" is improved comparing to differential direct coding (2d) algorithm. For improvements, we have partitioned dictionary into two parts as 'fix dictionary table' and 'dynamic dictionary table'.

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Evaluation of Hepatoprotective Potential of Leaf Extracts of Some Medicinal Plants Using HepG2 Cell line

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ABSTRACT

The present study was carried out to evaluate the hepatoprotective activity of *Adina cordifolia, Careya arborea, Cassia angustifolia, Hiptage benghalensis* and *Lannea coromandelica* against alcohol induced toxicity using HepG2 cell lines. Prior to the determination of hepatoprotective property, leaf extracts were subjected to the toxic dose study. The degree of hepatoprotection of extracts was determined by measuring cell viability percentage by MTT assay. The preliminary phytochemical analysis of leaf extracts was carried out by qualitative analysis. HepG2 cells were pretreated with the different concentrations (below toxic dose) of leaf extracts for 72 hours followed by alcohol intoxication. Results revealed that *Cassia angustifolia* ethanolic leaf extract pretreated HepG2 cells show 92% cell viability compared to the standard silymarin pretreated HepG2 cells which showed 80% cell viability. Out of the other plants tested, *Adina cordifolia* exhibited the significant hepatoprotection. The bio-efficacy study confirms the presence of promising secondary metabolites of hepatoprotective nature in leaf extracts of *Cassia angustifoliaand Adina cordifolia*.

KEY WORDS: ALCOHOL TOXICITY, HEPATOPROTECTION, CASSIA ANGUSTIFOLIA, MTT ASSAY, HEPG2 CELLS.

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INTRODUCTION

Plants as a source of medicine can be traced back over five millennia to written documents of the early civilisation of India and China. Plants have been frequently used to treat various ailments and some of the traditional medicines are still part of habitual treatment of various maladies. Popular observations on the usage and efficacy of medicinal plants contribute to the disclosure of their therapeutic properties so that they are frequently prescribed even if their chemical composition is not fully known. At global level, use of medicinal plants has significantly supported primary health care (Maciel et al., 2002)The herbal products today symbolise safety in contrast to the synthetic pharmaceutical products which are regarded unsafe for humans and environment. Alcohol abuse and alcoholism are the very serious current health and socioeconomic problems throughout the world. Alcohol induces a number of adverse metabolic changes in liver. Excessive consumption of alcohol for a long time leads to the steatosis, alcoholic hepatitis and cirrhosis resulting in weight and volume change of liver, (Kumar and Cotran, 2003 Londonkar et al., 2015 Sharma et al., 2016 Saha et al., 2019).

Alcohol Liver Disease (ALD) is one of the most serious consequences of the chronic alcohol abuse and is the second leading cause of death among all liver diseases (Rehm et al., 2003) .Liver cirrhosis, the culmination of the illness, is one of the main causes of the mortality in western countries (Fernández-Checa et al., 1993) .Researchers have found in the studies on animal models that liver injury in chronic alcoholics is due to oxidative stress which leads to fibrosis, impaired function of liver and increases apoptosis (Schuppan et al., 1995). Excessive alcohol intake elevates reactive oxygen species (ROS) production and this enhanced ROS production results in cell damage and death causing serious health concerns. One of the important characteristics of alcohol induced liver injury is an impaired vitamin A nutritional status (Ronis et al., 2004).

Studies in human HepG2 cell lines have shown that ethanol is cytotoxic and is apoptotic in nature predominantly in liver (Ibrahim et al., 2008). There are no satisfactory remedies available for liver diseases; hence search for effective hepatoprotective drugs from natural products is continued, (Wu and Cederbaum, 1999 Alla et al., 2014 Saha et al., 2019). Natural products play an important role in health care rehabilitation programmes worldwide. Medicinal plants are the significant source of hepatoprotective drugs. It has been reported that about 170 phytoconstituents isolated from 110 plants belonging to 55 families do possess hepatoprotective activity (Panda et al.,2006).Only a small number of traditionally used medicinal plants have been scientifically evaluated for the hepatoprotective property, (Alla et al., 2014). In our study, an attempt has been made to screen *Adina cordifolia, Careya arborea, Cassia angustifolia, Hiptage benghalensis* and *Lannea coromandelica* for hepatoprotective activity against ethanol induced toxicity using HepG2 cell lines.

MATERIAL AND METHODS

Fresh and healthy leaves of *Adina cordifolia, Careya arborea, Cassia angustifolia, Hiptage benghalensis* and *Lannea coromandelica* were collected from in and around the University of Mysore campus. The collected leaves were thoroughly washed under running tap water and shade dried in the laboratory. Dry leaves were ground to coarse powder by laboratory grinder and stored in air tight containers for further use.25 grams of dry leaf coarse powder was filled in a thimble separately and extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, ethanol and methanol in Soxhlet extractor for 48 hours. The solvent extracts were concentrated under reduced pressure and were stored at 50C in vials for further use. Leaf extracts were dissolved in 1% DMSO for bio-efficacy evaluation. HepG2 (Normal Human Liver Cell Line) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of HepG2 cell line were sub cultured as monolayers in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine Serum) and penicillin (100 µg/ml) (Trease and Evans, 1983).

Prior to the screening of hepato protective activity, toxic dose studies of leaf extracts and silymarin were carried out by MTT assay (3-(4,5-dimethylthiazole-2 yl)-2,5 diphenyl tetrazolium bromide). The IC50 value of ethanol was also calculated by MTT assay (Santhosh et al., 2007). HepG2 cells were subcultured, after 72 hours medium supernatant was flicked off, cell monolayer was trypsinized and the cell count was adjusted to the 1.0 x 105 cells/ml. To each well of the 96 well plate 200 µl of diluted cell suspension

(1x105 cells approximately) was added and the plates were incubated for 72 hours at 370C and 5% CO2 for 24 hours. After 72 hours when monolayer of cells was formed, medium supernatant was flicked off and the 200 µl of DMEM containing leaf extracts in concentrations below toxic dose was added to each well containing cell monolayer. The plates were then incubated for 72 hours at 370C and 5% CO2. This boosting of cells with the different extracts is also known as pre-treatment protocol. After 72 hours, the medium containing extracts was flicked off and DMEM containing ethanol at 150 mM concentration was added to the each well containing extract and silymarin pretreated cell monolayers. The plates were again incubated for 72 hours at 370C and 5% CO2.

After 72 hours of incubation, medium containing ethanol was flicked off and 100 µl of 5% MTT reagent in DMEM was added to each well and the plates were again incubated at 370C for 3 hours. After 3 hours, the supernatant was discarded and 100 µl of solubilisation solution (1% DMSO) was added to each well and the plates were gently shaken in gyratory shaker to solubilise the formed formazan (Kamel et al., 2010).The absorbance was then measured by using microplate reader at 630 nm and the percentage of growth inhibition was calculated using formula given as under:

Percentage growth inhibition =(Mean OD of the individual test group)/(Mean OD of control group)x100

All the assays were carried out in triplicates and the data analysis was carried out by using SPSS, DMRT. Preliminary phytochemical analysis of leaf and leaf callus extracts was carried out by the method described by (Sharma et al., 2016).

RESULTS AND DISCUSSION

Cytotoxic study of leaf extracts of all selected plants was carried out to standardize extract concentrations and evaluate their hepatoprotective activity. Results revealed that *Cassia angustifolialeaf* extracts above 225 µg/ml concentration is toxic to the HepG2 cells where the cell viability percentage was reduced to 40%. MTT assay for cytotoxicity of standard drug silymarin revealed that concentration above 75 µg/ml is toxic to the cells and hence 75 µg/ml was used as the test concentration for the subsequent study. IC50 value of ethanol was reported to be

Table 1. The cell viability percentage of the HepG2 cells pre-treated with leaf extracts of selected plants

Plant name	The cell viability percentage of the HepG2 cells pre-treated with leaf extracts of selected plants				
	Conc. (µg/ml)	Chloroform	Petroleum ether	Ethyl acetate	Ethanol
Adina cordifolia	100	58	57	60	68
	200	64	62	64	72
Careya arborea	100	56	58	52	56
	200	59	60	58	68
Cassia angustifolia	100	65	68	72	89
	200	71	70	74	92
Hiptage	100	54	62	65	70
benghalensis	200	56	66	68	72
Lannea	100	55	60	60	65
coromandelica	200	58	63	65	68
Silymarin	75	81			
Ethanol (Toxicant)	150 mM	50			
Control	Only cells	100			

*All the MTT assays were repeated thrice. Each value represents Mean±S.D. Statistical analysis done by DMRT (P≤0.5)

150 mM (0.69% ethanol. Out of the different extracts, polar solvent extracts like aqueous, ethanol and methanol pretreated cells showed significant viability percentage after intoxication with ethanol. HepG2 cells pretreated with leaf extracts showed a dose dependent increase in percentage viability. The cell viability ranged from 65% to 92% in leaf extracts. The maximum cell viability percentage (92%) was reported in C. angustifolia ethanolic leaf extract pretreated HepG2 cells at 200 µg/ml. The cell viability percentage of HepG2 cells pretreated with leaf extracts is presented in Table 1. Preliminary phytochemical analysis of leaf extracts revealed the presence of Alkaloids, flavonoids, saponins, phytosterols, phenols, terpenoids, triterpenoids and sterols. The study was aimed to evaluate the hepatoprotective property of leaf extracts of some ethnomedicinal plants. Drug, whether synthetic or natural, if consumed in excess dose damages the liver cells.

This is the reason; toxic dose study of drug is carried out. In the present study, cytotoxic study of leaf extracts was carried out by MTT assay before the extracts were subjected to the hepatoprotective activity. The principle involved is the cleavage of tetrazolium salt into its blue coloured derivative formazan; the process takes place in the inner mitochondrial membrane where an enzyme succinate dehydrogenasec leaves the tetrazolium salt. Only the living cell mitochondria reduce the MTT to coloured formazan, therefore the concentration of the dye is directly proportional to the number of metabolically active cells. The MTT assay revealed that concentration of leaf extracts above 225 µg/ml kill 60% of the cells. Toxic dose studies of the plant extract lays the foundation for the formulation of any type of herbal remedy (Babbar et al., 2011).

In the present investigation, ethanol was used as hepatotoxicant. Ethanol induced live injury is encompassed by wide spectrum of lesions, the most characteristic being alcoholic steatosis (fatty liver), alcoholic hepatitis, alcoholic fibrosis and cirrhosis (Kamali et al., 2016). Results revealed that ethanol treated groups of HepG2 cells showed a drastic decrease in the cell viability when compared to the HepG2 cell pretreated with the leaf extracts. Increase in the percentage of cell viability in pretreated HepG2 cells indicates that the cells get boosted up upon treatment with extract and does not allow oxidation to take place upon intoxication with ethanol. In our study, HepG2 cells pretreated with leaf extracts showed percentage viability more than that of positive control silymarin. The cell viability relies on the structure of membrane and any damage to cell membrane causes leakage of the cellular enzymes and consequently a cell death. Results revealed that HepG2 cells pretreated with leaf extracts show a dose dependent increase in the cell viability. The dose dependent cytoprotection has been reported in other plants like Cassia roxburghii (Kanchana and Jayapriya, 2013), *Polygonum multiflorum* (Londonkar et al., 2015), *Andrographis paniculata* (Sivaraj et al., 2011) and *Rumexvesicarius*, Kanchana and Jayapriya, (2013) (Arulkumaran et al., 2009).

Described in their study that the increase in the cell viability percentage maybe a consequence of membrane stabilisation boosted by phytochemicals and they further demonstrated that the plant extracts elevate the tissue antioxidant defence enzymes and thus tackles oxidative stress. Several researchers have concluded that the antioxidant activity of plant extract is generally attributed to the presence of phenolic compounds (Ibrahim et al., 2008) .Preliminary qualitative phytochemical studies revealed the rich presence of phenolic compounds in leaf and leaf callus extracts which could possibly be attributed to hepatoprotective nature of the extracts. The chemical constituents of leaf extracts may have interrupted the reaction of ROS with cell proteins and nucleic acids and thus prevented the formation of adducts by acting as scavengers and thereby stabilising the cell membrane resulting in increased cell viability. Our inference is in concurrence with the earlier studies carried out by (Santhosh et al., 2007), (Sharma et al., 2016) (Kamel et al., 2010) (Patil et al., 2011; Thabrew et al., 1997).

CONCLUSION

Ethanol induces production of reactive oxygen species (ROS), leading to huge oxidative stress which damages the liver cells. Leaf extracts of selected medicinal plants have shown the dose dependent hepatoprotection against ethanol. The results suggest the presence of active phytoconstituents in leaf extracts which strengths antioxidant defense in cells and thus minimizing the chances of production of free radicals. Furthermore, phytochemical analysis needs to be carried out to isolate and characterize

the bioactive compounds from leaf extracts with hepatoprotective activity and for further authentication pharmacological studies also need to be carried out.

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Authors' Contribution Kh: Tahamtan and Dr. M.S Sharada developed ideas and drafted the manuscript. Dr. M.S Sharada provided the required facilities. Kh. Tahamtan conducted the experiments. Kh .Tahamtan and Dr. M.S Sharad had contributed to the design and analysis of data. Authors contributed to revise the manuscript and approved the final version for publication.

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Geological Source of Fluoride in Fluoride Endemic Region of Gaya District, Bihar, India.

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ABSTRACT

The present study reports about the fluoride containing minerals present in the rock and soil samples collected from the fluoride endemic region of Gaya district of Bihar. Fluoride was measured in 77 water samples collected from fluoride endemic villages and 69 samples contained fluoride > 1.5 mg/L. A positive correlation was found between pH and fluoride (r=0.24), suggesting geogenic contamination of fluoride into groundwater. X-ray diffraction (XRD) analyses of rocks and soil samples were done. Results showed the presence of biotite in the rock and soil samples, suggesting that F might be leaching from biotite into the groundwater.

KEY WORDS: BIOTITE; FLUORIDE; HEALTH EFFECTS; XRD ANALYSIS.

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INTRODUCTION

Fluorine occurs in many common rock-forming minerals, including fluorite CaF_2, which occurs in both igneous and sedimentary rocks. Fluorine is the most abundant halogen in the sedimentary rocks. Clastic sediments can contain higher level of fluorine, while biogenic and chemical sediments may contain from <5 to >800 mg/ kg Fluorine (Wedepohl, 1978). The average Fluorine content of rocks is: 1000 mg /kg in alkalic rocks, 400 mg /kg in intermediate rocks, dropping to 100/ mg /kg in ultramafic rocks deficient in SiO_2 (Wedepohl, 1978). Fluorine is released as fluoride (F) during the process of weathering. F is released into the groundwater from F bearing minerals such as fluorite, fluoroapatite, biotite, amphinole, montmorillonite and some micas weathered from igneous and sedimentary rocks (Adimalla, 2018). F has the tendency to complex with other ions. In groundwater F may easily dissolve and form complexes with Ca^{2+}, Al^{3+}, Fe^{3+}, PO_4^-. Excess intake of fluoride (maximum permissible limit recommended by WHO=1.5 mg/L) can cause dental and skeletal fluorosis (Rao et al., 2017; Li et al., 2018; Adimalla and Quain, 2019).

However, the exposure dose of F may also depend on the amount of water intake which further depends on the local climatic condition, type of food and its preparation methods (Khan et al., 2004; Ranjan and Yasmin, 2015). Most states in India have their groundwater contaminated with fluoride and around 62 million people and 6 million children are facing health problems (fluorosis) due to consumption of F contaminated drinking water (Susheela, 1999; Jacks et al., 2005; Adimalla and Venkatyogi, 2018). Water quality assessment of Gaya district, Bihar was done between 2012-2014 and three villages were found to be fluoride endemic (Ranjan and Yasmin, 2012). Health survey was also conducted and the residents of these villages were found to suffer from dental, skeletal and non-skeletal fluorosis (Ranjan and Yasmin, 2012). Anomalies were also found in the thyroid function (Yasmin et al., 2013) and haematological parameters (Yasmin et al., 2014).

A detailed investigation found around 50% of fluoride intake was through drinking water, while the rest 50% came through food crops grown locally and irrigated with the same fluoride rich groundwater (Ranjan and Yasmin, 2015). Analysis of composition of rock and soil samples can reveal the source of fluoride in the groundwater of the region. A study on this aspect has not been conducted so far. Therefore, XRD analysis of rock and soil was done to confirm the presence of F contributing minerals.

MATERIAL AND METHODS

Gaya district is located at 84.4 ° E to 85.5 ° E Longitude and 24.5 ° N to 25.1° N Latitude and has total area of 487607.83 sq km. Gaya experiences subtropical climatic condition with extremely hot summer (maximum temperature shoots to 460C) and cold winter (mercury drops to as low as 40C). Average rainfall during rainy season is approx 338.4 mm. According to Adimalla and Venkatyogi (2018) higher evaporation and less precipitation rate in arid and semi arid region can increase F accumulation in groundwater. Till now there is no provision for rain water harvesting. The greater part of Gaya district is occupied by the Gangetic alluvium, but older rocks are found, chiefly in the south and east. These rocks are mostly composed of foliated gneiss, a subdivision of the Archean system which contains the oldest rocks of the earth's crust (O'malley, 2007). The south east corner of district is situated in the middle of rich mica belt. The foliated gneisses of oldest formations would have undergone maximum weathering. Weathering and leaching of fluoride bearing minerals lying in the joints, fractures and faults of such rocks under alkaline environment leads to the enrichment of fluoride in the groundwater (Raju et al., 2009). Similar findings were also reported by Sumalatha et al., (1999).

F in water samples were measured by using Fluoride Ion selective electrode Orion 9690 BNWP with PCD 650 cyber scan portable meter (range 0–500 mg/L, calibrated at 0.1, 1.0, 5.0, 10.0, 20.0 mg/L). Ion strength adjusting buffer (ISA I) was added to the samples before the measurement of F. Similarly, F concentration was estimated in the soil and crop samples as described previously (Ranjan and Yasmin, 2015).Rock and soil samples were collected from the F endemic areas. Soil surface was dug at the depth of 10 cm and sample was stored in clean plastic packets. The Rock samples were collected after breaking it with the help of hammer. Samples were brought to the lab and were grinded in MP100 ball mill, Retsech, Germany to get fine powder. Stainless steel jars and balls were used for making the fine powder. XRD analyses of the powdered samples

were done with the help of Miniflex 600 XRD, Rigaku, Japan. The X- Ray source was Cu filament. 2θ range was 50-800. Scan rate was 10/min. The step size was 0.02. Goniometer radius was 240 mm. The graph obtained was sent to IIT Mumbai for analysis with the help of PAN analytical software.

RESULTS AND DISCUSSION

An account of F level in groundwater, soil, food crops and major human health problems in F endemic areas are shown in Table 1. According to Wu et al., (2019) correlation plots provide geochemical factors for F accumulation in groundwater. Correlation matrix of physico-chemical parameters shows a positive correlation between pH and F (r=0.24) (Table 2). Similar results were reported by Narsimha and Sudarshan (2017) and Narsimha and Ranjitha (2018). A negative correlation was found between F and Ca (r=-0.38) which clearly indicates that the presence of high calcium results in lowering F concentration by precipitations. Similar findings were reported by Li et al., (2018) and Adimalla et al., (2018). Water soluble fluoride (WSF) of the soil of F-endemic area was high. Similarly, WSF and Total fluoride (TF) of crops grown in fluoride endemic areas were also high (Table 1).

Red and black rocks are predominant in F endemic villages of Gaya district. XRD pattern of black rock, red rock and soil samples are shown in Figure 1, 2 and 3 respectively. The graphs indicate the predominance of biotite in the rock and soil samples. Detailed mineral composition of black rock, red rock and soil samples are presented in Tables 3, 4 and 5 respectively. A positive correlation between pH and F in the water samples of fluoride endemic areas suggests that alkaline nature of water promotes leaching of fluoride from the rocks in these areas. The alkaline water most probably mobilizes fluoride from rocks with precipitation

Table 3. Fluoride concentration in groundwater, soil and vegetation of F endemic areas and related health complaints

Villages	F Level in Water (mg/L)		F in Soil (mg/kg)		F in Food crops (mg/kg)			Health Complaints (%)				
	Range	Mean	Water Soluble F	Total F	Water Soluble F	Total F	Sample size	Dental Fluorosis	Skeletal Fluorosis	Dental Caries	Gastro-Intestinal Problems	Joint Pain
Bhupnagar	1.7-7.2 (N=22)	3.5± 0.2	2.5±0.3 (N=19)	154.6± 13.6	1.4±0.1 (N=48)	6.3± 0.5	N=141	99 (67%)	41 (29%)	42 (29%)	88 (62%)	90 (63%)
Bhaktauri	1.1-5.1 (N=29)	2.3± 0.1	2.3±0.3 (N=18)	157.1± 15.8	3.3±0.7 (N=29)	10.4± 1.4	N=160	102 (63%)	65 (40%)	26 (16%)	90 (56%)	95 (59%)
Dhaneta	0.6-6.2 (N=26)	2.7± 0.3	2.4±0.3 (N=19)	149± 10.9	1.8±0.5 (N=33)	8.3± 1.1	N=133	97 (72%)	73 (54%)	48 (36%)	51 (38%)	80 (60%)

Figure 1. XRD Pattern of Black Rock Sample

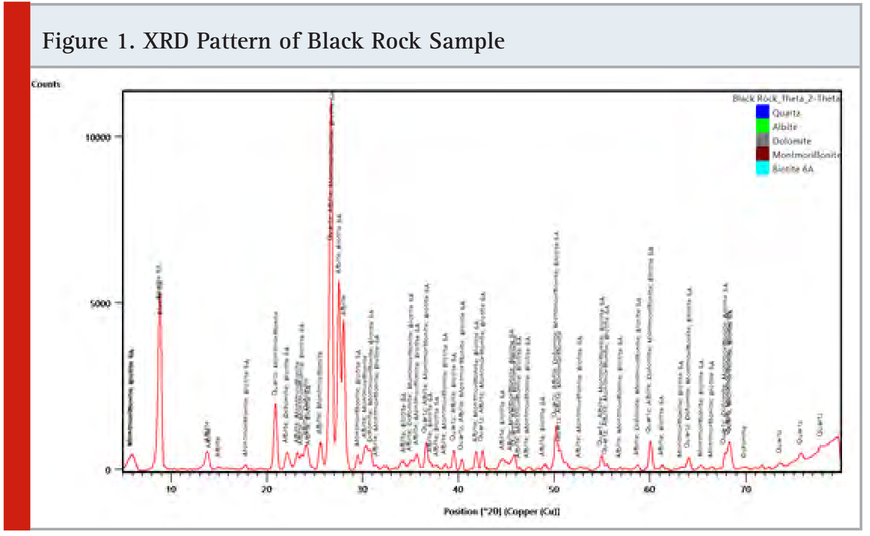


Table 2. Correlation between physico-chemical parameters in the F endemic region (N= 77)

	Turbidity	*pH*	*EC*	*TDS*	*Fluoride*	*TH*	*Calcium*	*Iron*	*Mg*
Turbidity	1								
pH	0.04	1							
EC	-0.23	0.28	1						
TDS	-0.24	0.29	0.99	1					
Fluoride	-0.14	0.24	0.06	0.05	1				
TH	-0.12	0.24	0.40	0.40	-0.33	1			
Calcium	0.17	-0.27	-0.11	-0.09	-0.38	0.40	1		
Iron	0.23	-0.22	-0.47	-0.44	-0.03	-0.10	0.22	1	
Mg	0.23	0.00	0.08	0.09	-0.29	0.09	0.33	0.15	1

Table 3. XRD analysis of Black rock

Reference Code	Score	Compound Name	Scale Factor	Chemical Formula
98-016-2490	54	Quartz	0.982	O2 Si1
98-008-7654	39	Albite	0.286	Al1 Na1 O8 Si3
98-017-1523	21	Dolomite	0.066	C2 Ca1 Mg1 O6
98-016-1171	13	Montmorillonite	0.049	H1 Al2 Ca0.5 O12 Si4
98-002-4167	33	Biotite 6A	0.618	H1 Al1 F1 K1 Mg3 O11 Si3

Table 4. XRD analysis of Red rock

Reference Code	Score	Name Compound	Scale Factor	Chemical Formula
98-016-2490	59	Quartz	0.986	O2 Si1
98-009-0142	39	Albite	0.171	Al1.02 Ca0.02 Na0.98 O8 Si2.98
98-016-1171	13	Montmorillonite	0.033	H1 Al2 Ca0.5 O12 Si4
98-009-8154	30	Biotite 1M	0.303	H1.47 Al1.92 F1. 98 Fe2.59 K2 Mg3.15 Mn0.09 O21.47 Si5.98 Ti0.27
98-020-2423	45	Microcline (maximum)	0.198	Al1 K0.95 Na0. 05 O8 Si3

of calcium carbonate because the solubility of fluorite (CaF2) increases with an increase in NaHCO3 rather than with other salts (Handa, 1975; Saxena and Ahmed, 2001). High level of fluoride in the soil may be due to geologic origin or due to irrigation with fluoride contaminated water. Similarly, WSF and Total fluoride (TF) of crops grown in F endemic areas were also high due to irrigation of crops with fluoride rich water. Fluoride was measured in the following crops: wheat (*Triticum aestivum*), rice (*Oryza sativa*), pigeon pea *(Cajanus cajan)*, chickpea *(Cicer arietinum)*, mustard *(Brassica nigra)* and vegetables including potato *(Solanum tuberosum)*, spinach *(Spinacia oleracea)*, tomato *(Solanum lycopersicum)*, coriander *(Coriandrum sativum)*. The XRD analyses indicate the predominance of biotite in the rock and soil samples. Furthermore, XRD peaks also showed the presence of Quartz, Albite, Dolomite, Montmorillonite and Microcline. Biotite is a common phyllosilicate mineral within the mica group, with the approximate chemical formula K(Mg,Fe)3AlSi3O10(F,OH)2. Biotite contributes upto 3400 ppm of fluoride into groundwater (Deer et al., 1992). In metamorphic terrain, high fluoride concentration in groundwater is due to the dissolution of Biotite, which may contain fluorine at the -OH sites of their octahedral sheet (Nordstorm et al., 1989; Li et al., 2003; Dharmagunawardhana, 2004). F mainly accumulates in aquifers from fluoride bearing minerals found in igneous and sedimentary rocks. These are the main source of fluoride contamination in groundwater, because longer contact time between rock and water with alkaline activities results in leaching of fluoride in fractured zones (Narsimha and Sudarshan, 2018). High concentration of F in groundwater results from dissolution of biotite because the position of fluorine in the octahedral sheet increases its capacity to dissolve in groundwater (Nordstorm et al., 1989; Li et al., 2003; Subba

Table 5. XRD analysis of soil

Reference Code	Score	Compound Name	Scale Factor	Chemical Formula
98-015-6196	68	Quartz	0.956	O2 Si1
98-009-0142	39	Albite	0.179	Al1.02 Ca0.02 Na0.98 O8 Si2.98
98-002-4167	34	Biotite 6A	0.295	H1 Al1 F1 K1 Mg3 O11 Si3
98-003-4787	31	Microcline (intermediate)	0.162	Al0.99 K0.94 Na0.06 O8 Si3.01

Figure 2. XRD Pattern of Red Rock sample

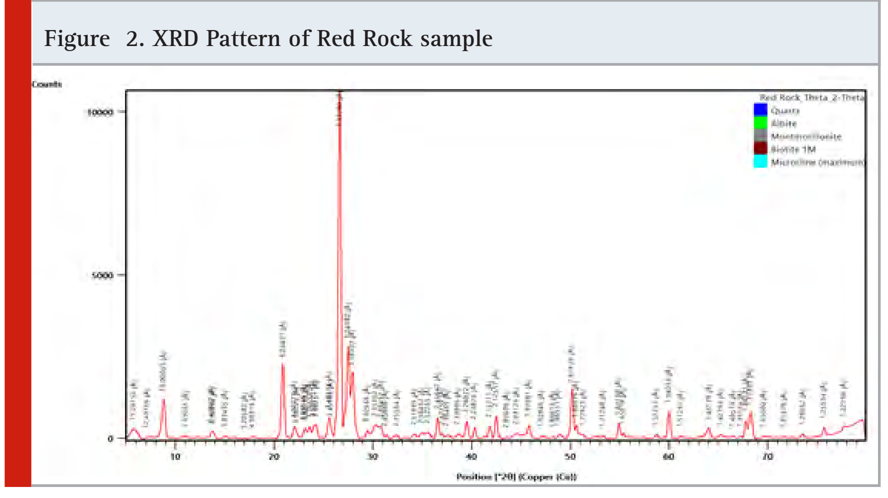
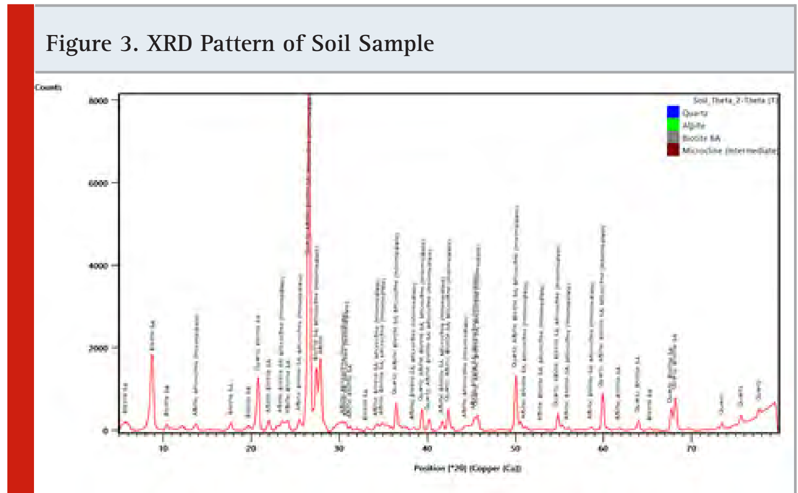


Figure 3. XRD Pattern of Soil Sample



Rao and Devdas, 2003; Chae et al., 2006; Msonda et al., 2007). In the rocks, biotite appears as tiny grains, providing large contact area with ground water and this enhances their solubility (Deutsch 1997). Various studies report that mineral rocks like syenites, granites, granite gneisses, quartz monzonites, granodiorites, felsic and biotite gnessis contributes high level of fluoride into soil and groundwater, (Handa, 1975; Apambire et al., 1997; Chae et al., 2007). The process of weathering of rock releases fluoride in soil and groundwater through anion exchange (OH- for F-) (Rankama and Edgington 1946; Abdelgwad et al 2009). According to Chae et al (2007), presence of biotite mineral in rocks may alone contribute to F concentration above 4mg/l. It may be due to similarity of ionic radii between F- and OH- group (Evans, 1995; Brownslow, 1996).

The fluoride contamination in groundwater in Gaya district is not due to anthropogenic origin as there are no industries in and around. Rather the contamination is due to geogenic origin. The inhabitants of the F endemic region of Gaya are underprivileged partly because the area is a Naxalite hit belt. They are economically poor (monthly income per family is INR 1750/ USD 25) and their main occupation is farming and firewood collection from nearby forests to sell in the market. As they spend most of their time in the field, therefore their water intake (hence, F intake) is high. The F exposure dose of the inhabitants has been discussed earlier (Ranjan and Yasmin, 2015). Public Heath and engineering department (PHED) of Bihar Government has installed filtration units in the F endemic areas for safe drinking water, but regular maintenance of such units are required. Provision for alternative safe water for irrigation and household purposes should also be made through supply from neighbouring F non-endemic regions. Another probable solution to the problem faced by fluorotic inhabtants of the area is diet counselling and diet editing (Ranjan and Yasmin, 2015; Susheela and Toteja, 2018)

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Antagonistic Activity of Lactic Acid Bacteria Against Common Enteric Pathogens Isolated from Milk and Milk Products and Evaluation of their Probiotic Attributes

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ABSTRACT

In the present study we have evaluated the antagonistic spectrum of 8 isolates of *Lactobacillus* against common enteric pathogens followed by auto-aggregation, co- aggregation and cell surface hydrophobicity. Isolate C9 showed antagonistic activity against all test species namely *Bacillus subtilis, Bacillus cereus, Salmonella enteric, Shigella flexneri, Streptococcus pneumonia , Staphylococcus aureus , Staphylococcus epidermidis, Pseudomonas aeruginosa, E. coli, Clostridium perfringens, Listeria monocytogens* and yeast *Candida albicans* that were obtained from IMTECH, Chandigarh. G4 showed highest zone of inhibition against *Listeria monocytogens* (20mm) while C28 exhibited highest zone of inhibition against *Shigella flexneri.* Similarly P37 showed against *Salmonella enteric* (24mm); C9 showed highest zone of inhibition against *Pseudomonas aeruginosa* (20mm) and E. coli (22mm). All the 8 *Lactobacillus isolates* exhibited the remarkable inhibitory effects against all test pathogenic strains with variable spectrum of inhibition. C9 showed highest auto-aggregation ability (91.6%) and co- aggregation activity against all the pathogens. Amongst all the eight isolates C9 showed noticeable higher hydrophobicity, hence the results suggest that the isolates *Lactobacillus* may be used as natural bio-preservatives in different food products and also to extent the shelf life of food products.

KEY WORDS: PATHOGENIC BACTERIA, LACTOBACILLUS, ANTAGONISTIC SPECTRUM, BIO-PRESERVATIVES, SHELF LIFE.

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INTRODUCTION

Increasing number of food borne pathogens and their entero- toxins has been concerned as an important research topic in the field of food safety and regulatory agencies (Kermanshahi and Qamsari, 2015). Infectious diseases caused by food borne pathogens which cause food spoilage such as *Escherichia coli* O157: H7, *Salmonella*, *Listeria monocytogenes* and *Campylobacter* cause various illness and death due to diarrhea (CDC, 2013; CDC, 2014; Dickinson and Surawicz, 2014). Commonly used antibiotics are efficient in limiting the growth of food borne pathogens, but led to expansion of bacterial resistance to antibiotics have been reported by Oroojalian et al., 2010; Andersson et al., 2010 and Campana et al., 2017 therefore the demand of new type of efficient and safe antimicrobial compounds are increasing (Gaspar et al., 2018; Bah et al., 2019). LAB's are potential microorganisms associated with fermentation. These are non spore forming gram positive bacteria, widely distributed in nature. Lactic acid is the main product, produced as they ferment carbohydrates primarily glucose in the raw materials to produce various metabolites which give food its unique flavor and increase nutritional value of the fermented food, which are not present before (Ren et al., 2017).

LAB as natural preservatives has a wide range of antimicrobial effects (Østergaard et al., 2014 and Mejlholm and Dalgaard, 2015) against many food borne pathogens (Sharma et al., 2017; Yang et al., 2017 and Zhang et al., 2018), widely used as bio-preservatives, extend shelf life which controlled food borne pathogens and has found application in many industries and also in various commercial purposes (Adeyemo et al., 2018). The mechanism underlying the activity of LAB strains against bacterial pathogens is due to the production of bacteriocin, organic acid, ethanol, hydrogen peroxide, diacetyl and reuterin , which stimulate immune system and modulate intestinal microbiota (Tesfaye, 2014 ; Kang and Im, 2015; Bah et al., 2019). LAB can prevent the adhesion of pathogens by competing for binding site on intestinal epithelial cell, reduced the colonization of pathogens and thereby preventing the onset of infection (Wang et al., 2018; Gao et al., 2019). Acid production is one of the mechanism by which LAB inhibit pathogens, bacteria are inactivated as the pH gets lower (Guo et al., 2017 and Wemmenhove et al., 2017). Theory of weak acids are also important, lipophilic when not dissociated; thus enter a bacterial cell through the plasma membrane and decompose into ions in a high pH environment which cause acidification of the cytoplasm (Gao et al., 2019).

Acidification alter the cell metabolism by damaging enzymes, inhibit protein synthesis, destroy genetic material, interrupt nutrient absorption and damaging the structure and function of membranes and cell wall (Hu et al., 2017). Zhang et al., 2011 proposed that aggregation of acid ions in intracellular space is important in determining antibacterial action of organic acid. Bacteriocin are ribosomally synthesized, extraecllularly released bioactive complex peptides that have a bactericidal or bacterio-static effects on other species (Masuda et al., 2012 and Costa et al., 2019). Bacteriocins have similar mechanism as that of probiotics. Bacteriocins compete with pathogenic agents for adhesion sites on mucosa (Simova et al., 2008; Jandaik et al., 2013 and Costa et al., 2019).

Bacteriocins modify the surrounding environment by modulating the p^H or the oxidation-reduction potential that compromise the ability of pathogens to become established (Feliatra et al., 2018). Bacteriocin provides beneficial effects by stimulating the humoral and cellular immune response (Dhanasekaran et al., 2010; Sieladie et al., 2011 and Wang et al., 2018). Most of the produced bacteriocins by LAB are cationic peptides at a neutral pH, hydrophobic in nature and amphiphilic which contain 20 to 60 amino acids (Yang et al., 2012; Sari et al., 2018 and Costa et al., 2018). The activity of bacteriocin are related to these properties when acting on the cytoplasmic membrane where the positively charged proteins bind to negatively charged phospholipids that make up a part of the membrane of sensitive cells (Cotter et al., 2005; Kumari et al., 2008 and Yang et al., 2012). Bacteriocins act by creating pores in the membrane of the target cells that produces harmful effects like dissipation of proton motive force, ATP depletion and leakage of nutrients and

Table 1. Antimicrobial spectrum of Lactobacillus sp .against various food borne pathogens

Lactobacillus isolates	Diameter of zone of inhibition (mean (mm) ± SD) against different food borne pathogens											
	Bac. subtilis	***Bac. cereus***	***Staph. aureus***	***Staph. epidermidis***	***Strept. pneumoniae***	***Pseudo. aeruginosa***	***E. coli***	***Shig. flexneri***	***Sal. enterica***	***Cand. albicans***	***Clostr. perfringens***	**List. monocytogenes**
G4	17 ±0.8	12 ±1.2	15 ±1.8	17 ±2	16 ±0.8	14 ±0.5	19 ±1.7	17 ±0.5	n.z	15 ±1.2	18 ±2.1	20 ±0.5
C28	10 ± 1	11 ±1.5	12 ±1.1	15 ±1.3	18 ±1.5	17 ±1.1	n.z	20 ±1.5	10 ±2.1	17 ±1.5	16 ±0.7	15 ±0.6
C9	19 ± 1.2	17 ±1	18 ±1.5	11 ±1	22 ±1.1	20 ±1.2	22 ±1.4	19 ±0.8	18 ±0.7	19 ±1	20 ±0.5	23 ±1.2
c20	n.z	14 ±0.5	15 ±0.9	16 ±1.7	12 ±1.9	12 ±1.6	17 ±2.1	18 ±2	14 ±2.1	13 ±0.8	18 ±1.1	19 ±1.5
P37	16 ± 1.5	10 ±0.8	12 ±1.4	20 ±1.5	10 ±1.3	17 ±2	n.z	15 ±2.4	24 ±0.5	11 ±2.1	n.z	16 ±1
c12	18 ±2	n.z	16 ±1	15 ±0.7	11 ±2	13 ±2.5	15 ±1	11 ±1.7	18 ±1.6	12 ±1.7	14 ±1.2	11 ±0.6
b1	14 ±0.5	15 ±1.1	15 ±1.2	18 ±0.5	n.z	n.z	11 ±2.1	10 ±2.8	14 ±1	11 ±2.1	15 ±2.1	n.z
B23	15 ±1	n.z	15 ±1.7	16 ±2.5	12 ±2.6	n.z	10 ±0.7	16 ±1	13 ±0.6	n.z	10 ±0.9	19 ±0.7

metabolites (Costa et al., 2018 and Feliatra et al., 2018). The pore size varies from one bacteriocin to another bacteriocin according to their size, stability and conductivity (Jolly et al., 2002). Bacteriocin has bactericidal or bacteriostatic properties against spoilage and pathogens bacteria and is used as a bio-preservative in food products and also considered an additional safety measure to minimally processed products (Kumari et al., 2008; Hwanhlem et al., 2015 and Astó et al., 2019). Intake of probiotics stimulates the growth of beneficial microorganisms which simultaneously reduces the amount of pathogens, thus improving the intestinal microbial balance and lowering the risk of gastro-intestinal diseases (Fuller, 1989; Cross, 2002; Chiang and Pan, 2012; Molina et al., 2012 and Wang et al., 2018).

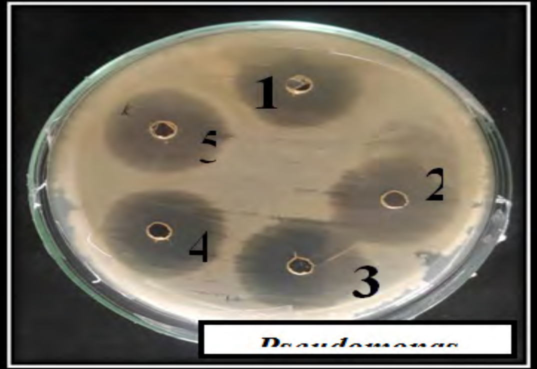
Probiotics are reported to also have anti-mutagenic, anti-carcinogenic, hypo-cholesterolemic, anti-hypertensive, anti-osteoporosis and immune-modulatory effects (Sieladie et al., 2011; Chiang and Pan, 2012; Shimizu et al., 2015; Wang et al., 2018; Colomer et al., 2019 and Tankoano et al., 2019). Lactic acid bacteria (LAB) is Generally Recognized as Safe (GRAS) by WHO, plays an important role in the process of fermentation of food by inhibiting spoilage bacteria and production of flavour, aroma, and texture of fermented food (Akkoc et al., 2011; Colomer et al., 2019 and Tankoano et al., 2019). The main objective of this study is to check the antagonistic activity of Lactic acid bacteria against food borne pathogens.

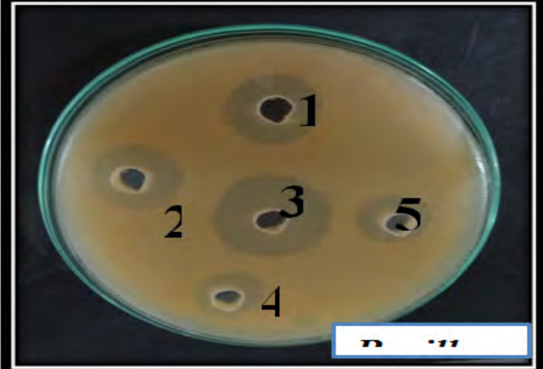
MATERIAL AND METHODS

Isolation of bacteriocin producing isolates: LAB strains were screened from traditional milk and milk products samples collected from different regions of Meerut. The bacteriocin producing strains from traditional milk products were isolated by the direct plating method (Coventry et al., 1997; Millette et al., 2007 and Gaspar et al., 2018) with slight modification: each sample were serially diluted ten folds with saline solution (0.9% NaCl). Aliquots (1ml) were plated onto MRS agar medium then incubated at 35 ±1°C under anaerobic conditions. Single isolates from these plates were each cultured in 10 ml of lactobacilli MRS broth for 24 h at 35 ±1°C and tested for antibacterial activity using agar well diffusion against pathogens i.e *Bacillus subtilis* MTCC441, *Bacillus cereus* MTCC430, *Salmonella enteric* MTCC1166, *Shigella flexneri* MTCC1457, *Streptococcus pneumonia* MTCC4673, *Staphylococcus aureus* MTCC7443, *Staphylococcus epidermidis* MTCC435, *Pseudomonas aeruginosa* MTCC4673, E. coli MTCC40, *Clostridium perfringens* MTCC 450, *Listeria monocytogens* MTCC 657 and yeast *Candida albicans* MTCC1637.

Identification of LAB: Gram's staining, endospore staining, catalase and oxide test, arginine hydrolysis and sugar fermentation (Arabinose, Cellobiose, Galactose, Glucose, Lactose, Maltose, Mannitol, Raffinose, Ribose, Sorbitol, Sucrose and Xylose) were conducted as a preliminary

Figure 1. Antagonistic activity of *Lactobacillus* isolates (A) against *Pseudomonas aeruginosa* (B) against *Bacillus cereus* (where1= G4, 2= C28, 3= C9, 4= c20, 5= P37) represent different isolates.



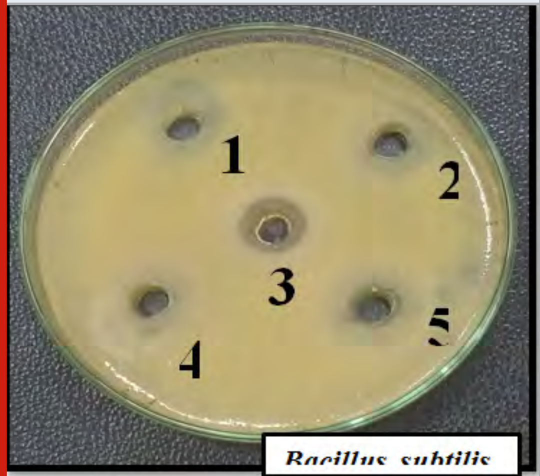


steps in characterization of Lactobacillus. In order to precisely identify the species various enzymatic activities- amylases, lipases, phytases, proteases and gelatinases were done followed by probiotics attributes (acidic, bile and pancreatin) and haemolysis.

Preparation of Cell free Supernatants (CFS): One liter of *Lactobacilli* MRS broth was inoculated with 10 ml of eight *Lactobacillus* species and incubated for 24 h at 35±1°C in bacteriological incubator (REMI). CFS was obtained by centrifugation at 10,000g for 10 min followed by neutralization of pH to 6.5 with the addition of 5 mol /l of NaOH. The resulting CFS was then filtered through a membrane filter (0.22 µm pore size).

Antimicrobial spectrum: The antimicrobial spectra of eight LAB against pathogenic bacteria such as *Bacillus subtilis* MTCC441, *Bacillus cereus* MTCC430, *Salmonella enteric* MTCC1166, *Shigella flexneri* MTCC1457, *Streptococcus pneumonia* MTCC4673, *Staphylococcus aureus* MTCC7443, *Staphylococcus epidermidis* MTCC435, *Pseudomonas aeruginosa* MTCC4673, E. coli MTCC40, *Clostridium perfringens* MTCC 450, *Listeria monocytogens* MTCC 657 and yeast *Candida albicans* MTCC1637 were examined by agar well diffusion assay (Schillinger and Lucke, 1989; Batdroj et al., 2006

Figure 2. *Antagonistic spectrum* of *Lactobacillus* isolates against Bacillus subtilis (where1= G4, 2= C28, 3= C9, 4= c20, 5= P37) represents different isolates.



and Gaspar et al., 2018). From Nutrient broth 100 µl of 24 h old cultures of the pathogenic bacteria were swab on the Müller-Hinton agar (MHA) plates and afterward wells were made using sterile cork borer. Wells were filled with 100 µl of the supernatant of each isolate and plates were incubated at 35±1°C for 24 h. The diameter of inhibition zones were measured and mean diameter for the inhibition zone was recorded, standard deviation was also calculated. The test was performed in triplicate (Putra et al., 2017).

Auto-aggregation assay: For auto-aggregation assay (Ramos et al., 2013), LAB's were grown in MRS broth for 18h at 35±1°C the cells were harvested at 9,000×g for 10 min at room temperature by centrifugation. The pellet washed twice in phosphate buffered saline (PBS) and re-suspended in PBS solution to a final concentration of about 108 cfu/ml. at this point, an absorbance was measured at 600nm (A0h) and then, 2 mL bacterial suspension were vortexed for 10s and incubated at 35±1°C for 5 h. After incubation, 1mL of the supernatant suspension was collected to measure the absorbance at 600 nm . Auto-aggregation (%) was calculated with the following equation:

$$\text{Auto-aggregation } (\%) = (1- At \;\; / \; A0h) \times 100$$

Where, At represents the absorbance at t = 1, 2, 3, 4 or 5 h and A0 is the absorbance at t = 0. The test was performed in triplicate

Co- aggregation assay: The co-aggregation potential of LAB isolates with different bacterial strains viz. *Bacillus subtilis* MTCC441, *Bacillus cereus* MTCC430, *Salmonella* enteric MTCC1166, *Shigella flexneri* MTCC1457, *Streptococcus pneumonia* MTCC4673, *Staphylococcus aureus* MTCC7443, *Staphylococcus epidermidis* MTCC435, *Pseudomonas aeruginosa* MTCC4673, *E. coli* MTCC40, *Clostridium perfringens* MTCC 450, *Listeria monocytogens* MTCC 657 and yeast *Candida albicans* MTCC1637, was examined. LAB and the test pathogenic bacteria were grown in MRS broth and nutrient broth, respectively, for 24h at 35±1°C. Bacterial suspension (108 cfu/ml) were formulated as described above as in the auto- aggregation in above method, equal volume

of LAB and pathogenic strains (1:1 v/v) were mixed and incubated at 35±1°C without agitation (Prabhurajeshwar et al., 2017). Absorbance, A600 of the mixture represent above, was supervised during incubation at 4 h, percentage of co-aggregation was calculated using the following equation:

Co-aggregation (%) = [(Apathogen +A LAB)] / 2-Amix (Apathogen +ALAB) /2×100

Cell surface hydrophobicity: The bacterial adhesion to hydrocarbons assay was performed according to the method Xu et al., (2009) with slightly modification to determine the cell surface hydrophobicity. Bacterial cells were suspended in phosphate buffered saline (PBS) pH 7.2 to 108 cfu /ml. Then, equal proportions of viable bacterial suspension and solvent (Xylene) were mixed by vortexing and incubated at 35±1°C for 10 min for temperature equilibration. To separate the mixture into two phases, the mixture was again vortexed briefly and allowed to stand for 5min. the aqueous phase was removed and its absorbance was measured at 600 nm. The results were reported as percentages according to the formula:

H% = [(Ao - A) / Ao] ×100

Where, Ao and A are absorbance before and after mixing with xylene, respectively.

Data analysis: All measurements of antagonistic activity, Auto-aggregation assay, Co- aggregation assay and Cell surface hydrophobicity were performed in triplicate. The data was expressed in the mean and standard deviation (± S.D) in triplicates.

Molecular Identification: The best selected isolates on the basis of their Auto-aggregation assay, Co-aggregation assay, Cell surface hydrophobicity and antagonistic activity against all the pathogens, was identified using 16s rRNA by Sanger dideoxy sequencing. The purified culture was sent for commercial sequencing. The sequence data obtained was compared using BLAST tool (Basic Local Alignment Search Tool).

Sequencing the DNA: The sequence data obtained was deposited to NCBI database with BLAST analysis for molecular identification.

RESULTS AND DISCUSSIONS

Total 8 isolates of LAB were isolated from curd, cow milk, buttermilk, goat milk and paneer samples based on their morphological, physiological and biochemical tests (Abdullah and Osman, 2010; Bisen et al., 2013; Guetouache and Guessas et al.,2015 and Kang et al., 2019) as per the guidelines of Bergey's Manual of Systematic Bacteriology (Hammes et al., 2009). All the 8 isolates were tested for antagonistic effect against common enteric pathogens *Bacillus subtilis* MTCC441, *Bacillus cereus* MTCC430, *Salmonella enteric* MTCC1166, *Shigella flexneri* MTCC1457, *Streptococcus pneumonia* MTCC4673, *Staphylococcus aureus* MTCC7443, *Staphylococcus*

Table 2. Mean auto-aggregation percentage of different isolates of *Lactobacillus* over a period of 5 h.

STRAIN	Auto-aggregation (%)				
	1 h	2 h	3 h	4 h	5 h
G4	11.3±1.3	36.6±1.4	61±1	70.6±1.6	75±1.1
C28	14.6±1.3	32.3±1.7	44.3±3	61.3±1.2	76.3±0.1
C9	26.6±2.4	54.3±1.4	67.3±0.1	83±2.1	91.6±1.2
c20	13.3±1.2	20±0.8	36.6±1.1	50±1.7	66±0.8
P37	16.3±1.2	27.6±2	44.6±1.4	52±1.1	61.3±2.6
c12	8±0.8	21.6±1.2	40±1	56.3±2	60.3±2
b1	15.4±2.1	20±1.5	35±0.8	44.3±1.3	50.5±2.1
B23	9.4±1.5	18.6±2	45.6±1.1	56.3±1.2	64.2±1.3

All values are mean ± standard deviation of triplicate

Table 3. Study of Co- aggregation ability (%) of LAB isolates with various bacterial pathogens

Lactobacillus isolates	Bacterial pathogens											
	Bac. subtilis	*Bac. cereus*	*Staph. aureus*	*Staph. epidermidis*	*Strept. pneumoniae*	*Pseudo. aeruginosa*	*E. coli*	*Shig. flexneri*	*Sal. enterica*	*Cand. albicans*	*Clostr. perfringens*	List. monocytogenes
G4	56.7 ±1.2	50.4 ±2.3	66.9 ±2.6	75.9 ±1.8	54.5 ±2.9	63.2 ±1.8	52.4 ±1.1	64.9 ±0.6	34.1 ±2.1	60.6 ±1.9	54.3 ±0.9	69.4 ±2.8
C28	51.4 ±2.6	55.6 ±2	60.5 ±1.9	61.3 ±2.8	72.9 ±1.1	70.3 ±2.6	32.5 ±2.4	86.2 ±0.5	50.3 ±1.9	79.8 ±2	64.8 ±1.9	66.2 ±2.6
C9	90.2 ±0.5	70.2 ±1.5	87.3 ±0.1	43.5 ±1	94.9 ±0.8	93.7 ±2.8	75.9 ±0.5	84.4 ±1.8	63.5 ±1.1	89.4 ±2.5	90.2 ±2.1	89.7 ±0.9
c20	45.4 ±1.9	33.3 ±2	55.6 ±2.1	65.2 ±1.7	50.5 ±1.1	75.4 ±0.8	89.5 ±2.7	66.2 ±0.4	76.2 ±3	88.3 ±0.8	60.6 ±1.5	72.1 ±2.5
P37	89.2 ±2	63.2 ±0.9	60.5 ±2.6	90.8 ±1.9	42.6 ±0.5	78.9 ±1.7	36.6 ±2.1	86.1 ±2.9	95.2 ±2.9	72.5 ±1.1	54.2 ±1.2	68.9 ±1.1
c12	50.7 ±2.8	42.8 ±1.1	80.2 ±1.8	75.2 ±2.2	65.4 ±2.6	55.6 ±1.5	68.5 ±1.3	78.7 ±2.4	85.8 ±0.1	70.9 ±1.5	78.1 ±3	60.8 ±2.4
b1	78.9 ±3	62.5 ±2.1	75.6 ±2.3	92.8 ±2.5	48.9 ±0.8	30.5 ±2.2	80.5 ±1.9	60.2 ±3	66.5 ±0.8	50.2 ±0.9	68.9 ±2.5	23.2 ±1.6
B23	72.3 ±0.9	65.4 ±1.9	70.8 ±1.7	78.5 ±1.1	33.5 ±2.2	26.5 ±1.2	52.6 ±2.9	68.6 ±2.2	60.4 ±0.5	58.2 ±1.9	45.8 ±1.8	80.2 ±0.9

epidermidis MTCC435, *Pseudomonas aeruginosa* MTCC4673, E. coli MTCC40, *Clostridium perfringens* MTCC 450, *Listeria monocytogens* MTCC 657 and yeast *Candida albicans* MTCC1637 (Table1). The antagonistic activity of LAB is due to the production of antimicrobial compounds such as lactic acid, ethanol, hydrogen peroxide (H_2O_2), diacetyl, reuterin, bacteriocin and biosurfactants (Cizeikiene et al., 2013; Guetouache and Guessas et al., 2015, Gaspar et al., 2018). Amongst all the 8 isolates only C9 showed antagonistic spectrum against all the pathogens (Table 1). All the 8 isolates shows antagonistic spectrum against Staphylococcus aureus, Staphylococcus epidermidis and Salmonella enteric. Except c20 all the species shows maximum zone of inhibition against Bacillus subtilis, highest by C9 (19mm), c12 (18mm), G4 (17mm) followed by P37, B23, b1 and least by C28 (Table 1; Figure 2). Except c12 and B23, All the LAB isolates showed antagonistic spectrum against *Bacillus cereus* (Figure 1(B)), similar results were reported by Nigam et al., 2012.

Except b1, all the isolates shows highest zone of inhibition against *Streptococcus pneumonia* by C9 (22mm), followed by C28 (18mm), G4 (16mm), B23 (12mm), c12 (11mm) and least by P37 (10mm). Against *Pseudomonas aeruginosa* highest antagonistic spectrum by C9 (20mm) followed by C28 and P37 (17mm), G4 (17mm), c12 (13mm) and least by c20 (12mm) (Table1; Figure 1(A)). Except C28 and P37 all showed antagonistic acitivity against E.coli Highest by C9 (22mm) and lowest by B23 (10mm). Except G4 all isolates showed zone of inhibition against Salmonella enteric. Against Candida albicans, highest zone of inhibition showed by C9 (19mm), C28 (17mm), G4 (15mm) followed by c20, c12 and lowest by P37 and b1 (11mm). Except P37, all showed maximum zone of inhibition against Clostridium perfringens. Highest zone of inhibition against Listeria monocytogens shown by C9 (23mm), G4 (20mm), C28 and B23 (19mm) followed by P37, C28 and c12. All the 8 isolates of LAB can be used as alternative in food preservatives and also replace chemical additives (Costa et al., 2018 and Hu et al., 2017). They also satisfy consumers' demands for fresh, healthy and safe food (Ahmad et al., 2017). Cotter et al., 2013 stated that the use of bacteriocin as food preservative meet consumers' demand for natural preservation and also considered additional safety measure to minimally processed products. Yang et al., 2014; Ahmad et al., 2017 and Kang et al., 2019 reported that bacteriocins were able to kill target microorganism by disrupting membrane integrity which little induce resistance, since their fragments do not interact also with target cells and the best potential solution for growing problems of microbial resistance to antibiotics. One of the most important prerequisite for the colonization of the host intestinal tract is the adhesion ability of probiotics to intestinal mucus and enterocytes has been also proposed as one of the important selection criteria for potential probiotics (Xu et al., 2009). Through in vitro model system adhesion ability of probiotics has been studied, which are commonly used in selection and assess of probiotics strains for in vivo studies (Baick and Kim, 2015).

For in vitro adhesion tests generally immobilized intestinal mucus and human enterocyte-like Caco3 cell cultures are mostly used but these methods are expensive and time consuming, (Andrabi et al., 2016) therefore, an auto- aggregation test together with cell surface hydrophobicity (CSH) have been extensively used for the preliminary screening and identification of potential adherent bacteria with suitable commercial (Zielinska et al., 2015). Bajaj et al., (2015) stated that Cell surface hydrophobicity by xylene partition has been used as an indirect test to estimate the adhere ability of probiotics to epithelial cells. The results of auto- aggregation assay are shown in table 2. C9 showed highest auto-aggregation ability of 91.6% followed by C28 (76.3%), G4 (75%), c20 (66%), B23 (64.2%), P37 (61.3%), c12 (60.3%) and least by b1 (50.5%). Prabhurajeshwar et al., (2017) stated that the auto- aggregation % increased with increased in incubation time and results were persistent. Baick and Kim, (2015) also reported 90% as the highest auto- aggregation % similarly Andrabi et al., 2016 documented 94.97 highest auto aggregation % at 37°C and at 25 °C 96.54% was documented as the highest auto-aggregation percentage. All the eight isolates were further tested for their co-aggregation ability with different pathogens viz., *Bacillus*

Table 4. Cell surface hydrophobicity

Lactobacillus isolates	Hydrophobicity (%)
G4	0.5±1.2
C28	51.4±1.2
C9	79.0±0.7
c20	5.2±0.5
P37	8.0±2.2
c12	37.1±2.5
b1	15.6±1.8
B23	19.1±0.2

subtilis MTCC441, *Bacillus cereus* MTCC430, *Salmonella enteric* MTCC1166, *Shigella flexneri* MTCC1457, *Streptococcus pneumonia* MTCC4673, *Staphylococcus aureus* MTCC7443, *Staphylococcus epidermidis* MTCC435, *Pseudomonas aeruginosa* MTCC4673, E. coli MTCC40, *Clostridium perfringens* MTCC 450, *Listeria monocytogens* MTCC 657 and yeast *Candida albicans* MTCC1637 (Table 3). Excellent co-aggregation potential was exhibited by all the isolates except some isolates showed low co-aggregation like G4 against Salmonella enteric i.e 34.1%, c20 against Bacillus cereus (33.3%), P37 and c12 against E. coli (36.6% and 32.5%), b1 against *Pseudomonas aeruginosa* and Listeria monocytogens (30.5% and 23.2%) and B23 against Streptococcus pneumonia and *Pseudomonas aeruginosa* however, with other pathogens it showed relatively batter co-aggregation (Table 3). Overall all the isolates had remarkable co-aggregation potential with pathogens. Isolates tested in this study showed a variable hydrophobicity with values ranging from 0.5% to 79% (Table 4).

Amongst all the eight isolates C9 showed noticeable higher hydrophobicity. Among the eight isolates, isolate C9 isolated from cow milk exhibit antagonistic activity against all the food borne pathogens and common enteric pathogens. Analysis of the 16S rDNA sequence of isolate C9 showed high homology with that of available strains of *Lactobacillus fermentum* (National Center for Biotechnology Information, NCBI database); thus, isolate C9 was identified as *Lactobacillus fermentum* and designated as *Lactobacillus fermentum* C9. The sequence has been submitted to GenBank under accession number MN421922.

CONCLUSION

Thus, it may be safely concluded that all eight isolates of Lactobacillus isolated in this study can be safely used as food preservatives.

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Physical, Microbial and Sensory Qualities of Cookies Produced from Composite Flour

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ABSTRACT

Cookies are produced from blends of chickpea, wheat, soybean, sorghum and oat flours, which is extremely important as there are important crops, which are good source of protein, minerals and trace elements. Chickpea flour was prepared from four different varieties i.e. two Kabuli varieties -Kripa (Phule G-0517) and RVKG- 101 and two desi- JAKI-9218 & JG-130. Blend was prepared by homogenously mixing chickpea with other flours in the proportions of 60:10 (60% chickpea & 10% of each other flours used to bake cookies). The study was carried out to evaluate the varietal effect of these blended flours on physical, microbial and sensory qualities. Bacterial and Fungal growth were evaluated using standard form. Consumer preference was also determined using a taste panel list. The main quality scores of bacterial count on the cookies from 0 to 90 days of four varieties ranged (0.28-14.03 x 10^4 and 0.21-8.19 x 10^4 cfu/100g) for total viable count on PDA & NAM. For fungal count on PDA & NAM medium ranges (0.06-0.85 x 10^7 and 0.03 -0.74 x 10^7 cfu/100g) were estimated. The counts were minimal and within acceptable limits. In sensory evaluation of cookies it was carried out in 20-35, 35-50 & 50-65 age groups. In all the age groups cookies made from flours of different varieties were found significantly different ($p<0.05$) exist in color, texture, taste and mouthfeel. Cookies made form kabuli gram had overall higher ranking than those made form desi gram varieties.

KEY WORDS: COOKIES, CHICKPEA FLOUR, PHYSICAL ANALYSIS, MICROBIAL, SENSORY.

ARTICLE INFORMATION

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INTRODUCTION

In the present day economic scenario, emerging globalization, consumers have changed the perception of their foods. Most of the consumers demand convenient food, ready to eat snacks which add to bulk and satisfy their appetite (Kumar et al., 2016). In this era of industrialization and technological advancement, the life style of people has changed (Udaybeer et al., 2018). The diet consumed by a vast majority of people is deficient in proteins, minerals etc. Therefore, one of the great challenges today is to develop inexpensive foods that are nutritionally superior and at the same time highly acceptable to intended consumers. The consumption of cereals and legumes all over the world gives them an important position in International Nutrition (Goliya et al., 2018).

Chickpea seed has high digestible protein and complex carbohydrate with low glycemic index and is relatively free from anti-nutritional constituents increased cancer prevention as well as protection against cardiovascular disease due to their dietary fiber content. A multigrain snack helps in high intake of fiber and health enhancing components (Regaee and Abddel Aal, 2006 Wang et al., 2010, Wallace et al., 2016, Parca et al., 2018, Raza et al., 2019). Wheat is the world's number one cereal supplying major portion of energy and protein in the Indian diets (Shewry and Sandra 2015). Soybean being the richest, cheapest and easiest source of best quality protein and fat and having a vast multiplicity of uses as food and industrial products is sometimes called a wonder crop (Rana et al., 2013). Oats are high in fibre, specifically beta-glucan, which reduces blood cholesterol level by increasing the excretion of bile in the body (Jenkins and Kendall, 2012; Varma et al., 2016; Grundy at al.,2018). Millets are being recognized as potential future crops because of relatively high dietary fiber, antioxidants, micro-nutrients, sulphur containing amino acids and essential fatty acids besides macro-nutrients. Particularly in the develop countries, there is a growing demand for gluten-free foods and beverages for people with celiac disease (Rao et al., 2017). Indians consume 42 per cent of the millets produced globally (Anonymous, 2007). India is the largest producer of many kinds of millets, which are often referred as coarse cereals (Michaelraj Shanmugam, 2013). The sensory evaluation of the product improves the consumer appeal which helps in marketing and dissemination of the product (Aziah et al., 2012).

Table 1: Basic formulation of cookies

Ingredients	Quantity in (gram)
Flour/composite flour	500 gm
Sugar	250 gm
Ghee/butter	250 gm
Eggs (White)	3 (Nos.) wt. 60 gm each
Baking Powder	10 gm

Figure 1. Cookies of four different gram varieties

Sensory evaluation helps customer to reduce risks, improve their product's appeal and enhance quality characteristics as a result of different process such as packaging, storage, new processing or changing ingredients (Bala et al., 2015). Microbiological changes are primary important for short-life products, whole chemical and sensory changes for medium to long life products. Microbial spoilage is the major problem causing deterioration in bakery products. It is caused mainly by molds and yeasts and occasionally by bacteria (Saranraj and Geetha 2012). The purpose of the study is to evaluate Shelf-life studies of products of chickpea cultivars through microbial load and to study the impact of selected recipes on various age groups -College girls and elders.

MATERIAL AND METHODS

Raw Material & cookies preparation: Four released varieties 2 *kabuli* and 2 desi of chickpea (*Cicer arietinum* L.) were procured from Department of

Plant Breeding and Genetics, Rafi Ahmed Kidwai College of Agriculture, Sehore while local grains of Soybean, Wheat, Sorghum and Oat were procured from local market. Butter, sucrose, baking powder etc. were purchased from grocery store.Cookies were prepared from the composite flour according to the method of (Giami et al., 2004) (Table-1, Fig.-1). The composite flour and baking powder are added to the sugar-butter-egg mass and mixed to get homogenous mass. Baking was carried out at 225^{0}C for 13 minutes. The biscuits were cooled at room temperature for 15 minutes. The cookies were allowed to cool for 30 minutes and stored in polyethylene bags before further analysis.

Determination of Physical properties: Physical properties of the cookies were determined according to AACC (2000) methods. Six cookies were weighed on an electronic (Mettler Toledo, India) weighing balance. The diameter was determined by placing six cookies edge to edge using a ruler. The cookies were rotated at an angle of 90^0 for duplicate readings. The height was measured by placing six cookies on top of one another using a ruler. The spread factor was expressed as the ratio of the diameter to the thickness of the cookies. All measurements were carried out in triplicate.

Sensory Evaluation: The sensory evaluation of the cookies samples was carried out for consumer acceptability and preference using 150 untrained and semi-trained panelists comprised of students, staff of agriculture and home science departments and different age group (20-35, 35-50 & 50-65) people. They were to evaluate the sensory properties based on Taste, Flavor, Crispiness, Appearance and Overall acceptability using a nine point Hedonic scale where 1 represents "extremely dislike" and 9 "extremely like" respectively.

Storage Studies/Microbial observation: For microbial estimation of cookies, PDA & NAM medium mixtures were sterilized in an autoclave at 121^{0}C for 20 minutes. The molted medium was poured in pre-sterilized Petri-plates. The samples were ground to fine powder and stored in polypropylene bags for 0, 30, 60 and 90 days. The ground powder of respective samples were spread on petri-dishes and incubated at 30 ± 20^{0}C temperature for 48 hours. The fungal and bacterial colonies were counted over PDA and NAM after 48 hours of incubation.

Statistical Analysis: Three replicated measurements were taken for each analysis and the results were expressed as the mean of values ± standard deviation. The significance of treatments were observed by calculating F ratio compared with Fisher's Table F value. Significance of difference were tested using the Duncan Multiple Range Test at 5% level of probability.

RESULTS AND DISCUSSION

Physical Parameters of cookies: Results of these studies indicate that there is a significant difference ($p < 0.05$) between diameter and thickness of cookies made from four different varieties, while, no differences were observed for spread factor. There was an increment in *kabuli* gram varieties by about 48% for diameter in cookies than desi gram varieties. The diameter of the cookies ranged from 40.00 to 73.00 mm with the mean of 58.50 + 3.60 mm. The diameter of cookies made from Kripa was the highest one (73 mm) as compared to the cookies of other three

Table 2. **Physical analysis of Cookies of gram varieties**

Variety	Kripa	RVKG 101	JAKI 9218	JG 130	General Mean	SEm	C.D. 5%
Diameter	73.00	67.00	54.00	40.00	58.50	1.04	3.60
Thickness	13.50	12.50	10.22	7.94	11.04	0.33	1.15
Spread factor	5.41	5.36	5.28	5.04	5.30	1.73	6.01

Figure 2. Physical analysis of cookies

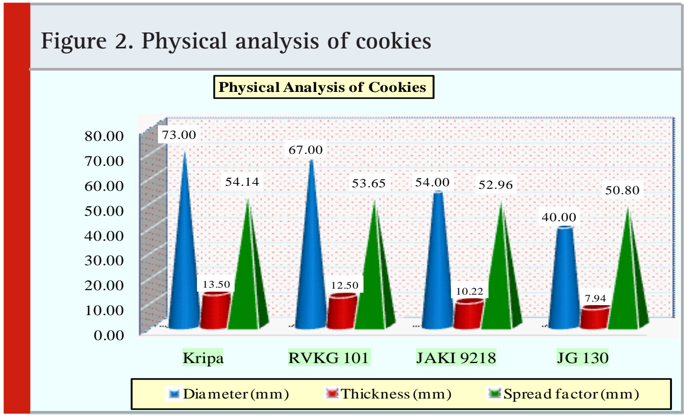


Table 3. Sensory Evaluation of cookies of different gram varieties

Age Grp.	Variety/ Parameter	Kripa	RVKG 101	JAKI 9218	JG 130	General Mean	SEm	C.D. 5%
20-35	Color	9.87	8.77	6.23	3.93	7.20	0.15	0.54
35-50		9.00	8.50	7.50	4.73	7.43	0.19	0.67
50-65		9.63	9.57	4.10	2.80	6.53	0.19	0.69
20-35	Texture	9.67	7.20	7.30	4.60	7.19	0.28	0.98
35-50		8.40	9.40	8.57	4.90	7.82	0.16	0.55
50-65		8.97	8.40	5.13	2.50	6.25	0.20	0.67
20-35	Taste	8.60	8.40	7.60	5.50	7.53	0.22	0.76
35-50		7.50	8.50	7.40	4.30	6.93	0.095	0.33
50-65		9.10	8.67	6.00	1.67	6.36	0.29	1.01
20-35	Mouthfeel	9.40	8.60	8.20	7.50	8.43	0.11	0.40
35-50		8.23	9.20	8.20	3.80	7.36	0.28	1.00
50-65		8.23	8.70	4.90	2.33	6.04	0.23	0.81
20-35	Overall Acceptability	9.38	8.24	7.33	5.38	7.59	0.082	0.28
35-50		8.28	8.90	7.92	4.43	7.38	0.12	0.44
50-65		8.98	8.83	5.03	2.33	6.29	0.087	0.30

varieties. The thickness of cookies made from the *kabuli* gram was in general significantly of higher magnitude than those made from desi gram varieties. However, in general, spread factor of cookies was higher in Kripa variety (5.41), but no significant differences were observed either in cookies made from different gram varieties (Table-2, Fig.-2). The increase in thickness of multi-grain cookies might be due to the fact that baking powder acts as chemical leavening agent. The carbon dioxide, ammonia gas and steam produced during baking process increases the thickness and crispness of cookies. Vishwakarma, (2016) and Goliya (2017) also reported the similar findings. The result of increase in diameter of composite flour cookies are in close agreement with the result of (Siddiqui et al., 2003) and (Kukade, 2017) who reported increase in diameter with increasing level of soy flour in biscuits. (Sanaa et al., 2006) reported that the substitution of wheat flour with 30% of barley, millet or sorghum whole grain meal had no significant effects on the quality (i.e. cookie height and diameter) of cookie products.

Sensory Evaluations of cookies: In all the age groups, cookies made from flours of different varieties were found significantly different for

Figure 3. *Sensory evaluation* of Cookies between 25-35 age groups

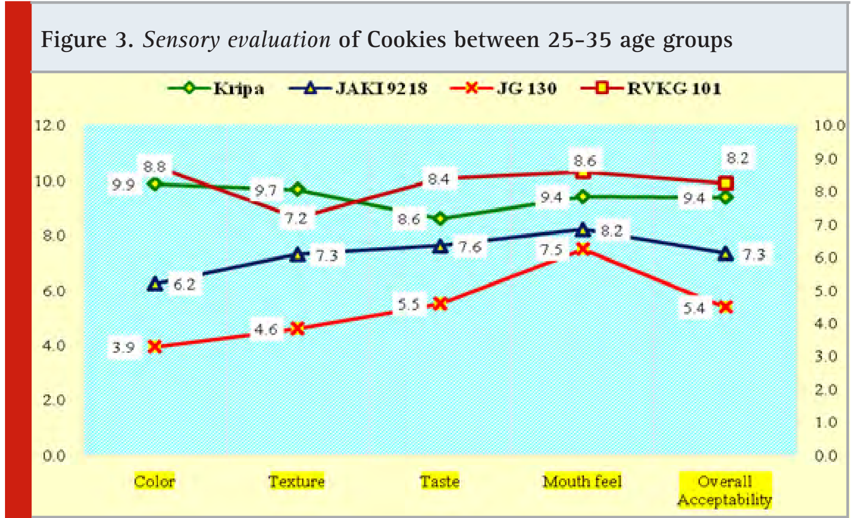
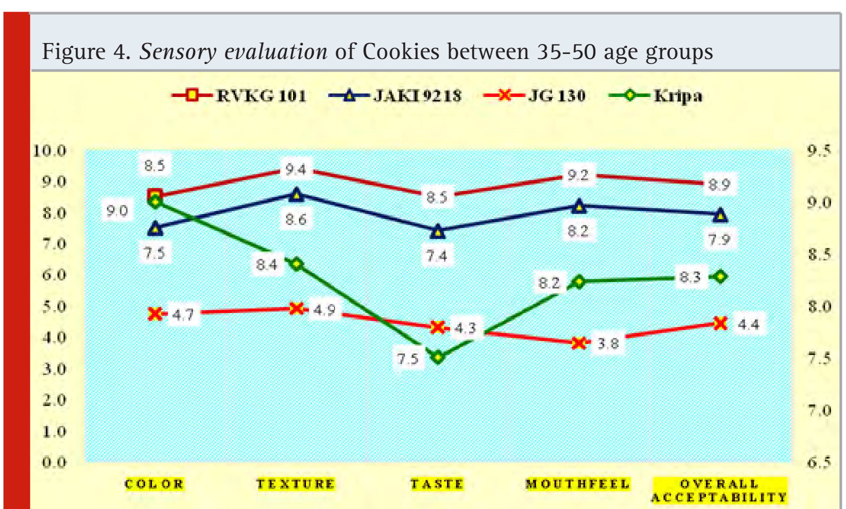


Figure 4. *Sensory evaluation* of Cookies between 35-50 age groups



color, texture, taste, mouth feel (Table- 3, Fig-3, 4 & 5). Cookies made from *kabuli* variety Kripa ranked first (9.38) closely followed by cookies made form RVKG 101 (8.24). The lowest ranked cookies were those made from the flour of JG 130 (5.38). Likewise, in the age group of 35-50 as well as of 50-65, the most liked cookies ranking highest point were those made from *kabuli* gram variety Kripa followed by RVKG 101 and to certain extent cookies of JAKI 9218. Cookies made form *kabuli* gram had overall higher ranking than those made form desi gram varieties. Similar findings have been reported by (Mridula et al., 2007). This finding was also agreed with the results of (Maha et al., 2012) who reported that addition of sorghum to wheat flour decreased sensory quality of biscuits. (Singh et al., 2000) reported decrease in taste score of biscuits containing 20 per cent defatted soy flour when stored at ambient condition. (Kukade, 2017) and (Vaijapurkar et al., 2013) and (Chavan, 2013) reported the similar results after supplementing pearl millet flour with wheat flour to prepare cookies. This kind of evaluation was also been reported of the cookies made from different blends of Wheat Flour Fortified with Termitomyces robustus flour by (Giwa et al., 2012) and cookies made from blends of wheat/cassava/cowpea flour by (Adekunle et al., 2014).

Microbial Study: The Bacterial population on PDA at 60 days was found lowest (3.75) on the cookies made of *kabuli* gram variety Kripa, while it was significantly higher on the cookies made from

Table 4. Association of prox. components with bacterial & fungal counts in cookies.

Proximate Character	PDA 60 days	PDA 90 days	NAM 60 days	NAM 90 days	PDA 60 days	PDA 90 days	NAM 60 days	NAM 90 days
		Bacteria				Fungal		
Protein	0.87	0.86	0.83	0.89	0.52	0.88	0.78	0.84
Crude fiber	0.97	0.99	0.99	0.87	0.64	0.87	0.92	0.91

Figure 5. *Sensory evaluation* of Cookies between 50-65 age groups

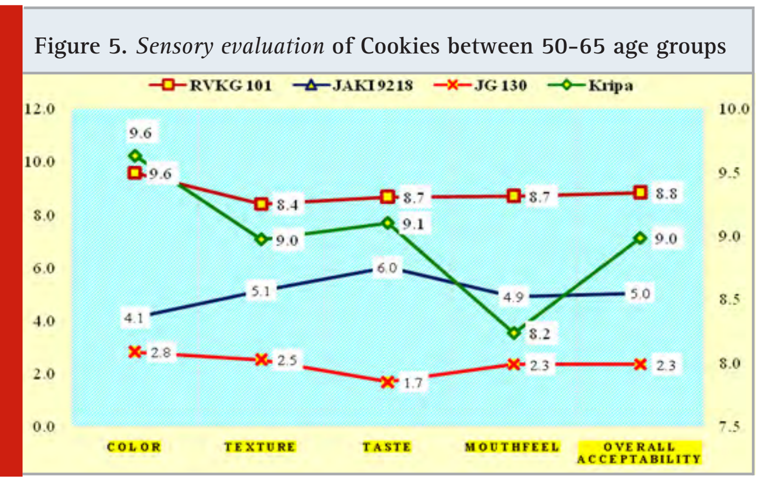
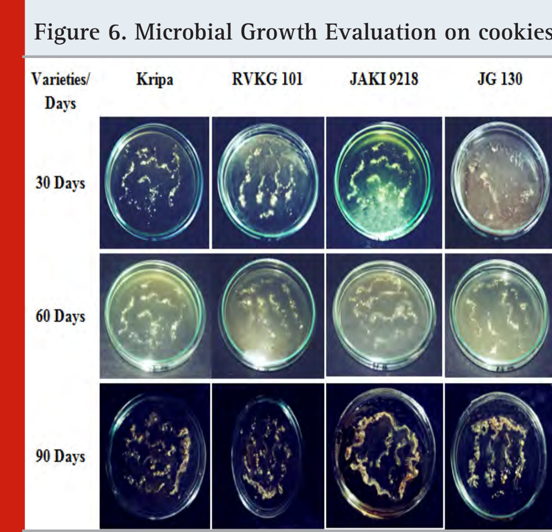


Table 5. Microbial (Bacterial & Fungal) population of Cookies of different gram varieties.

Variety	PDA- Bacterial(10^4cfu/100_g)				NAM- Bacterial (10^4 cfu/100_g)			
	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days
Kripa	0.32	1.71	3.75	12.43	0.28	0.55	2.15	7.18
RVKG 101	0.16	1.73	4.90	13.71	0.11	0.64	2.48	7.31
JAKI 9218	0.28	1.59	6.26	14.80	0.21	0.71	2.72	8.73
JG 130	0.35	2.38	6.98	15.19	0.22	1.14	2.88	9.52
General Mean	0.28	1.85	5.47	14.03	0.21	0.76	2.56	8.19
SEm	0.062	0.25	0.408	0.151	0.086	0.056	0.142	0.118
C.D.5%	0.214	0.87	1.41	0.524	0.299	0.196	0.492	0.409

Variety	PDA- Fungal (10^7 cfu/100_g)				NAM- Fungal (10^7 cfu/100_g)			
	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days
Kripa	0.03	0.07	0.18	0.60	0.00	0.11	0.36	0.61
RVKG 101	0.02	0.05	0.15	0.64	0.02	0.26	0.41	0.66
JAKI 9218	0.05	0.08	0.19	0.97	0.03	0.34	0.48	0.79
JG 130	0.13	0.20	0.39	1.19	0.06	0.46	0.59	0.91
General Mean	0.06	0.10	0.23	0.85	0.03	0.29	0.46	0.74
SEm	0.022	0.020	0.017	0.053	0.004	0.033	0.015	0.055
C.D.5%	0.076	0.071	0.059	0.183	0.014	0.116	0.054	0.192

Figure 6. Microbial Growth Evaluation on cookies



desi gram JG 130 (6.98). The linear growth was observed in 90 days also (Table-5 Figure-6). In the study of bacterial growth on NAM medium on cookies was also found to be significantly higher on 30,60 & 90 days on the cookies made from desi chickpea JG 130 while, it was lowest on the cookies made form *kabuli* chickpea Kripa. While, compared to other three varieties, fungal population of cookies of Kripa was found to be the lowest one. It has been observed that growth of fungus and bacteria on PDA & NAM media observed on cookies had significant preference from which they are prepared.

Cookies made from the flour of desi gram JG 130 are more prone to the higher bacterial population than those of products prepared from other three varieties because of their high protein and fiber content in the flour and cookies. The similar kind of finding has been reported by (Yousufu et al., 2016). Results show that the spoilage organisms grow faster in a medium that is highly nutritious. It indicated that the varietal differences exist in making the product suitable or unsuitable for the shelf life. In the present investigation, it has also been observed that the protein and fiber content of the cookies are positively correlated with the bacterial and fungal count at 60 & 90 days in both the media (Table 4).

CONCLUSION

Demand and consumption of snack foods is increasing day by day. Therefore, the monitoring of newly developed genotypes and advanced breeding material of chickpea for various quality characteristics should be emphasized and suitable genotypes be identified through chickpea improvement programs. Information on the quality of snacks made from newly developed varieties of chickpea is important so as to overcome resistance encountered by the plant breeder from the consumers, when new varieties are introduced in the market. The overall diameter & spread of cookies made from *kabuli* gram has larger appearance which favors its acceptability. In sensory evaluation, it is concluded that cookies & crackers made form *kabuli* gram varieties have higher acceptability by most of the age groups. The longevity because of least microbial growth on cookies or crackers made from the flour of *kabuli* gram is higher which increases its shelf life.

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A Survey on Internet of Things (IoT) Applications and Challenges for Smart Healthcare and Farming

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ABSTRACT

Nowadays Internet of Things (IoT) succeed a great attention from investigators, after all it becomes a big automation that contract a smart body being life, by allowing a means between article, machines and everything constant with persons. The Internet of Things (IoT) is a logically connected device and system which is composed of smart machines combined and communicating with other machines. Internet of Things have wide applications in agreement to bring immense value into our life. This paper focuses on Internet of things and their applications and challenges in certain areas of life sciences like environment, health and agriculture. In this paper we have presented how smart health care, environment and agriculture fields are benefitted from Internet of Thing structures..

KEY WORDS: IOT APPLICATIONS, SMART ENVIRONMENT, HEALTHCARE AND FARMING.

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INTRODUCTION

Internet of Things is a system of computing devices that are connected from each other. These computing devices should be power-driven as well as digital machines and these computing devices can transfer data over a network deprived of demanding human-to-human or human-to-computer communication. In IoT, Statistics transmission between one computing devices to another calculating device without personal communication. Essentially Internet of Things IoT strategies consist web-enabled smart devices and these devices made by surrounded processors, sensors and hardware (Tubaishat, 2018 Magsi et al 2018). These devices can communicate with each other, including those which can transfer data in distributed environments. The IoT helps people live and work smarter as well as have far reaching improvements. Internet of Things is must for employment as it conveys productions with an immediate look into how the companies' systems actually work effectively, distributing perceptions into all from the achievement of apparatus to stock chain and coordination actions, (Simitha et al. 2019).

Internet of Things is a class of software application program for action authority, the gathering of data in real time from remote locations to control apparatus and conditions. In 2008 the Internet of Things was born and in 2011 the market research company Gartner, included "The Internet of Things" technology in their research. Internet of Things is one of the biggest technologies of normal life. Internet of Things (IoT) is very useful for our daily life for example, smart toilet balances working in tandem with treadmill, delivering food preparation ideas to laptops or smartphone to make people healthy, (Zorzi et al 2017 Magsi et al 2019). Security devices monitoring homes, turning lights on and off as one enters and exits rooms. It helps and controls streaming of videos so one can check in while he is away. Clever estimation assistants engaging ones traditional ready-to-wears, order on command, making it a breeze to get fresh nutrition transported to ones entrance (Anggorojati et al 2012).The Internet of Things has many characteristics such as-

1. Internet of Things save time and money
2. Internet of Things can display occupational process
3. By using IoT users can access data from anywhere any time
4. Provide fast communication between connected devices
5. Improve quality of business
6. IoT provide high security
7. By using IoT users can take better decision for good business

The Internet of Things (Iot) Model: The Internet of Things is a device of connected manipulative methods, motorized and virtual machines, objects, animals or people which are well-found with exact dependent on and the competence to switch facts over a network without stressful human-to-human or human-to-pc interaction, (Xiao et al., 2014 and Xiao and Wang 2014 Magsi et al 2019). IoT devices are part of the advanced concept of home automation that can consist of lights, heating and air condition media and safety structures. Enduring assistances ought to consist of enthusiasm sources by means of frequently assure streamers and microelectronics are exaggerate (Magsi et al., 2019).

Process of Internet of Things: The Internet of Things movement twitches with the system like smartphones, smartwatches, digital applications like TV, Washing Machine which assist you to transmission with the Internet of Things floor (Lee, 2014 Razzak et al., 2017). There are 4 component of Internet of Things.

Sensors/Devices: Sensors or devices are a key element that recommendation you to acquire animate Statistics from the connecting weather. All this facts may also have destructive elevation of complication. It will be a simple temperature monitoring sensor, or it may be inside the form of the video corn.

Connectivity: All the imperturbable information is dispatched to a cloud base. The sensors have to be related to the cloud the usage of innumerable channel of transportations. These connection channel embody mobile or restraint networks, Bluetooth, WI-FI, WAN, and so forth.

Data Processing: Once that information is accidental, and it converts to the cloud, the software program convey approximately relate at the amassed data. This trade may be neutral inspection the infection, analyzing on techniques

like AC or reservoirs. However, it can every now and then also be very multipart like recollect materials, using pc estimation on cinematic.

User Interface: The cloth request to be appropriate to the end-user in a few resources which may be entire by harvest seizures on their earphones or sending them statement thru e-mail or textual content message. The consumer irregularly power need a border which unaffectedly audit their IOT machine. For instance, the person has a digital camera properly-appointed in his domestic. He desires to get admission to video broadcasting and all of the barley with the assist of an internet server.

Applications of Iot: There are some popular applications of Internet of Things:

1. Smart City
2. Smart Grids
3. Industrial Internet
4. Smart Retail
5. Smart Farming
6. Pharmaceutical
7. Insurance

Smart City: The Internet of Things attack new user-friendliness for cities to use data to charge gridlock, cut pollution, make improvement use of base and keep native perfect. Cities are energetic important advance, transporting primary challenge as they seek to continue viable, active and harmless credit for people to invigorate and work, (Zorzi et al., 2017).

Smart Grids: Smart community technology has the strength to prepare these days' towns for the following day's needs. With more than half of the arena's communal fixed in municipal centers, cities will want to research new suitability, solutions and structures to hold fabric walking for its humans, industries, and administrations.

Industrial Internet: The Engineering Internet is the combination and mixing of massive statistics, effective equipment and wireless networks with physical and modern-day fixtures, or contrariwise applying meta-stage networking achievement, to allotted systems.

Smart Retail: Today, peddle stores are automatically focusing on leveraging the attained mechanizations like cloud computing, mobiles, beacons, etc., to provide connected marketing services and better spending action to customers. For example, store holders are associate sensors in the key area of retail stores and connecting them to cloud through an appearance that donation real-time data examination related to products, sales, and customers from these sensors, (Kumar et al 2014).

Smart Farming: IoT based Smart Farming improves the entire Agriculture system by monitoring the field in real-time. With the help of sensors and interconnectivity, the Internet of Things in Agriculture has not only saved the time of the farmers but has also reduced the extravagant use of resources such as Water and Electricity. It keeps various factors like humidity, temperature, soil etc. under check and gives a crystal clear real-time observation.

Pharmaceutical: The pharmaceutical business is totally situated to enjoy the benefits of industrial IoT technologies. Between the manufacturing plants, supply chains, delivery routes and, of course, the patients themselves, there are many sources of data out there and an increasingly broad selection of tools for taking advantage of them (Castrucci et al 2012 Kumar and Patel 2014). Patients, medication businesses and supply chain operatives all have a lot to gain from compliant the IoT on a larger scale.

Insurance: Internet of Things (IoT) technologies enable insurance corporations to regulate hazards more exactly. Auto insurers, for example, have traditionally relied on indirect indicators, such as the age, address, and comfort of a driver, when setting premiums. Now, data on driver behavior and the use of a vehicle, such as how fast the vehicle is driven and how often it is driven at night, are available. Applications of such technology in countries where the market is already much more mature reveal that insurers can assess risk far more accurately this way. Networked devices also allow insurers to interact with their customers more frequently and to offer new services based on the data they have collected. In the insurance sector in particular, customers often engage exclusively with agents or brokers; direct customer contact has been limited to contract extensions and the handling of insurance claims.

Internet of Things Devices: Internet of Things can connect devices implanted in numerous systems to the internet. When devices/objects can signify themselves digitally, they can be

controlled from anywhere. The connectivity then helps us capture more data from more places, ensuring more ways of increasing effectiveness and refining protection and Internet of Things safety (Vignesh 2017). Internet of Things devices are basically smart devices which have support for internet connectivity and are able to interact with the other devices over the internet and grant isolated access to a user for supervision the device as per their essential. These IoT devices are purely integrated with high definition technology which makes it possible for them to communicate or interact over the internet smoothly and can also be managed and controlled remotely when required. Approximately there are around 7.62 billion humans on our planet, but to your surprise, by the year 2021 with an increasing graph of IoT devices, there may be around 20 billion IoT smart devices up and running with an increase in the demand of 5g network.

There are several top devices in the market. Smart Mobiles, smart fridge-freezers, smart watches, smart fire alarm, smart door lock, smart motorbike, medical sensors, fitness trackers, smart security system etc., are few examples of IoT products. For example, a user reaches home and his car communicates with the garage to open the door. Once inside, the thermostat is previously familiar to his preferred infection, and the lighting is set to a lower intensity and his chosen color for relaxation, as his pacemaker data indicates it has been a stressful day (De et al. 2011 Gubbi et al 2013, Lee et al 2014 Hossain et al 2015, Xhiao and Wang 2014 Magsi et al 2918).

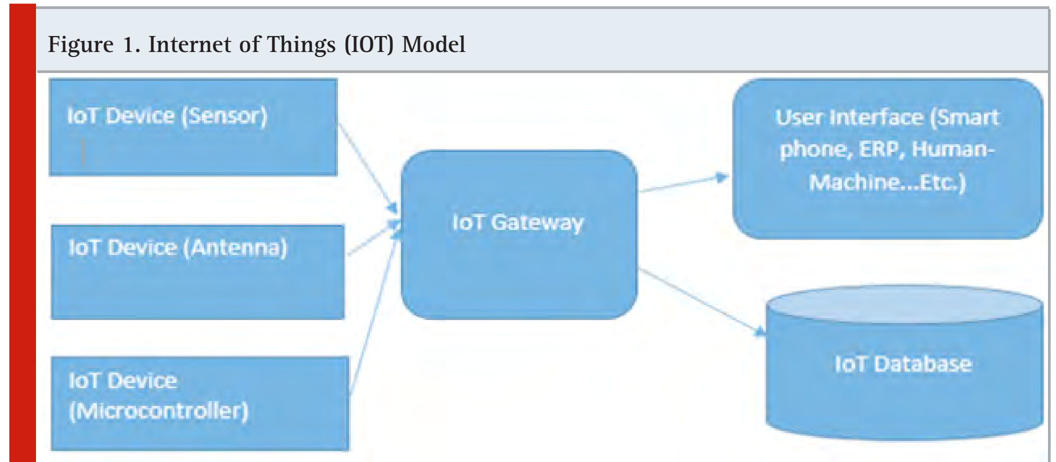
The networking, communication and connectivity protocols used with internet-enabled devices principally depend on the unambiguous IoT application deployed (Kovalchuk et al., 2019). Just as there are many different IoT applications, there are many different connectivity and transportations possibilities. Communications protocols contain CoAP, DTLS and MQTT, among others. Wireless protocols include IPv6, LPWAN, Zigbee, Bluetooth Low Energy, Z-Wave, RFID and NFC. Cellular, satellite, Wi-Fi and Ethernet can also be used. A number of challenges can hinder the successful deployment of an IoT system and its connected devices, including security, interoperability, power/processing capabilities, scalability and availability. Many of these can be addressed with IoT device management either by adopting standard protocols or using services offered by a vendor.

Internet of Things Standards:
There are some standards of Internet of Things
LiteOS: LiteOS is a Unix-like operating device for Wi-Fi sensor networks. LiteOS cares smartphones, wearables, shrewd production applications, clever homes and Internet of Vehicles (IoV). The operating machine also serves as a smart device improvement platform.

6LoWPAN (IPv6 over Low-Power Wireless Personal Area Networks): 6LoWPAN is an open standard defined via the Internet Engineering Task Force (IETF) (Xhiao and Wang 2014). The 6LoWPAN general permits any low-strength radio to communicate to the internet, such as 804.15. Four, Bluetooth Low Energy and Z-Wave (for home automation).

ZigBee0: ZigBee0 is a low-strength, low information-fee Wi-Fi network used in particular

Figure 1. Internet of Things (IOT) Model



in industrial settings. ZigBee is primarily based on based totally the IEEE 802.15.Four standard (Zorzi, et al., 2017 Li et al 2018). The ZigBee Alliance created Dotdot, the commonplace language for IoT that permits smart gadgets to work securely on any network and apprehend each other.

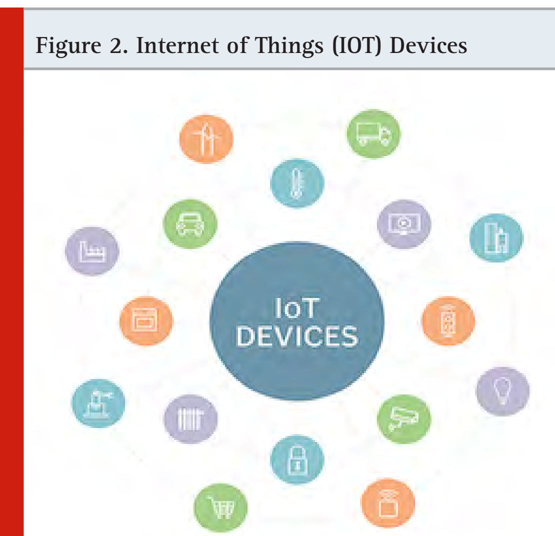
OneM2M: OneM2M is a machine-to-machine service layer that can be embedded in software and hardware to connect devices. The global standardization body, OneM2M, was created to develop reusable standards to enable IoT applications across different verticals to communicate (Kumar and Patel 2014).

DDS (Data Distribution Service): DDS (Data Distribution Service) changed into evolved via the Object Management Group (OMG) and is an IoT well known for real-time, scalable and high-performance machine-to-device verbal exchange.

AMQP (Advanced Message Queuing Protocol): AMQP is an open source posted general for asynchronous messaging by twine. AMQP permits encrypted and interoperable messaging among organizations and applications. The protocol is used in patron/server messaging and in IoT tool control, (Neisse et al.. (2014) Mohan et al 2014 Kovalchuk et al., 2019).

CoAP (Constrained Application Protocol): CoAP is a technique deliberate by the IETF that has the same opinion how low-strength calculate-uncommon approaches can stimulate within

Figure 2. Internet of Things (IOT) Devices



the net of things. LoRaWAN (Long Range Wide Area Network): LoRaWAN is a protocol for wide location networks, it's planned to sustenance huge networks, including smart towns, with hundreds of low-power techniques.

Internet of Things in Agriculture: Agriculture plays an essential function in manufacturing and for livelihood. The Internet of Things has the ability to convert the ways we stay in the global. We have extra-efficient industries, greater related automobiles, and smarter towns, a lot of these as additives of an integrated Internet of Things device. Smart farming via the usage of Internet of Things technologies will help farmers to lessen generated wastes and enhance productivity. That can come from the quantity of fertilizer that has been utilized to the wide variety of journeys the farm automobiles have made. So, clever farming is basically a hello-tech device of developing food this is smooth and is sustainable for the masses (Anggorojati et al., 2012; 2014 Magsi et al 2018 Li et al., 2018).

It is the induction in addition to the utility of modern-day ICT (Information and Communication Technologies) into agriculture. The use of IT and objects like sensors, self-sufficient automobiles, automated hardware, manipulate structures, robotics, and so forth on this method are key components, (Chen et al., 2014 Kumar et al.2014, Kumar 2017). IoT applications help farmers to collect data regarding the location, well-being, and health of their cattle. This information helps them in identifying the condition of their livestock. Such as, finding animals that are sick so, that they can separate from the herd, preventing the spread of the disease to the entire cattle. The feasibility of ranchers to locate their cattle with the help of IoT based sensors helps in bringing down labor costs by a substantial amount.

CONCLUSON

Internet of things (IoT) is a new expertise which adds many utilities to connect the things and human beings through the internet. Internet of things can afford some elegant attractive applications in making healthcare, transportation, insurance and agriculture work more efficiently. However, various factors like security, privacy and data storage also need to be considered. Though Internet of Things can improve the product, but

associated security problems have also to be considered.

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Design and Prediction of Absorption, Distribution, Metabolism, Excretion and Toxicity Properties of Drugs for Dengue fever Using In-silico Approaches

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ABSTRACT

Dengue fever is caused by *Dengue Virus* (DENV) and it has become a serious threat to human lives. This study was aimed at in-silico drug design and prediction of Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of potential hits against dengue fever using nonstructural protein 3(NS3). NS3 plays a vital role in the life cycle of dengue virus. It is a target for the development of specific antiviral inhibitors. The 3D structure of *Dengue Virus* 4 NS3 Helicase in complex with ssRNA is available at Protein Data Bank. It contains Glycerol as a ligand. Compounds were selected from DrugBank, PubChem, ChemSpider and they were screened along with Glycerol using PyRx-Virtual Screening software for Structure based drug design (SBDD) and compared using similarity score. Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties were determined by using online tool Danish Quantitative Structure-Activity Relationship (QSAR) database. The Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) analysis and docking results revealed 12 compounds as potential leads.

KEY WORDS: ADMET, *DENGUE VIRUS* 4, NS3 HELICASE – SSRNA COMPLEX.

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INTRODUCTION

Severe dengue fever (otherwise called Dengue Haemorrhagic Fever) was first perceived during the 1950s during dengue epidemics in the Philippines and Thailand. Today, it affects most Asian and Latin American nations and has turned into a main leading cause of hospitalization and death among children and adults in these regions(Bollati et al., 2010; Qaddir, Rasool, Hussain, & Mahmood, 2017). As indicated by World Health Organization (WHO), dengue is a mosquito borne viral infection that has quickly spread in all locales as of late. This virus is transmitted by female mosquitoes mainly of the species *Aedes aegypti* and, to a lesser degree, *Aedes albopictus*("*Dengue Bulletin*," 2016). This mosquito additionally transmits chikungunya, yellow fever and zika disease. Dengue is boundless all through the tropics, with local variation in risk affected by rainfall, temperature and impromptu rapid urbanization("Dengue Bulletin," 2016). There are 4 distinct, however firmly related, serotypes of the virus that cause dengue (DEN-1, DEN-2, DEN-3 and DEN-4)(Mukhopadhyay, Kuhn, & Rossmann, 2005; Wilschut, 2010).Recuperation from disease by one gives lifelong immunity against that specific serotype. Be that as it may, cross-immunity to the other serotypes after recovery is only partial and temporary.

Subsequent infections by other serotypes increase the risk of developing severe dengue(Kuhn et al., 2002). It is an acute infectious disease characterized by headache, pain in various parts of the body, fever, rash, leucopenia and so on(Alvarez, Laura, Ezcurra, Fucito, & Gamarnik, 2005).The instance of dengue fever has expanded in ongoing time in different geographic areas with the distribution of the viruses and the mosquito vectors and it has led to build pandemic activity, the development of hyper endemicity and the emergence of dengue hemorrhagic fever in new geographic regions(Whitehead, Blaney, Durbin, & Murphy, 2007). Dengue viruses enter and replicate with the help of certain structural and nonstructural proteins in cells of mononuclear phagocyte to further increase the *Dengue Virus* infection in these cells(Mazzon, Jones, Davidson, Chain, & Jacobs, 2009).Dengue infection can cause fever of different severity. At the point when the distinctive strains of dengue virus connect with the immune system of different person it can cause complex interaction resulting in conditions such as dengue fever or dengue shock syndrome. At present there is not any treatment available, in the form of vaccine or drug. The control of mosquitoes has shown only limited success(Qi, Zhang, & Chi, 2008). Therefore, there is a need to develop novel drugs for *Dengue fever*. One of the most promising target for development of novel drugs is the dengue non-structural protein 3 (NS3), the biggest and most highly conserved dengue proteins(Ackermann & Padmanabhan, 2001).The NS3 protein of *Dengue Virus* is a multifunctional protein having molecular weight of 69 kDa, provided with helicase, protease, and nucleoside 5-triphosphatase (NTPase) activities(Ago et al., 1999). The computer-aided drug design is a specialized branch that utilizes computational strategies to create drug-receptor interactions (Bissantz, Folkers, & Rognan, 2000). The computer-aided drug design methods are greatly dependent on the tools of bioinformatics, applications and databases. Since the structure of the NS3 is available, structure based drug design approaches are best suited for novel drug design to identify potential hits and lead molecules(Bissantz et al., 2000).

Most of drug hopefuls fail in Phase III clinical trials after many years of research. The disappointment is due to toxicity or problems with metabolism. The ADMET properties of drugs are Absorption, Distribution, Metabolism, Excretion and Toxicity in other words bioavailability and bioactivity. However these properties are typically measured in the wet lab, they can likewise be normal ahead of time with the assistance of bioinformatics software and tools. The range of bioinformatics apparatuses and web indexes (search engines) are available for this work (Bissantz et al., 2000; Iskar, Zeller, Zhao, Noort, & Bork, 2011). Once potential lead molecules have been identified the next stage is to fine tune their structural and ADMET properties. This commonly includes the arrangement of changes in accordance with the essential and secondary structure of the compound. The procedure can be improved by utilizing software that explores related compounds to the lead candidate(Song, Lim, & Chuantong, 2009).

MATERIAL AND METHODS

Target Identification: *Dengue Virus* contains structural and nonstructural proteins. Among all these proteins non-Structural protein named *Dengue Virus* 4 NS3 Helicase in complex with ssRNA (figure 1) was identified as target. The structure of the protein molecule was downloaded in from Protein Data Bank (PDB) with PDB ID 2JLU. The experimental detail shows, Resolution[Å]: 2.04, R-Value: 0.197 (obs.) and R-Free:0.242. Drug Library:Online chemical databases throughout the web were employed to obtain compounds. This mixes were screened which had related structures and properties with Glycerol, (propane-1,2,3-triol) using similarity score. Such properties like Common and IUPAC name of Compounds, Melting and Boiling Point of Compounds, pKa Value, Polarizability, Molecular Weight, Solubility, Density have been already reported(Dias, Filgueira, & Jr, 2008; Fernando et al., 2008).

Docking: Structure files such as .sdf/.mol/.pdb of those chemical compounds were downloaded from the chemical compound database like PubChem, DrugBank, ChemSpider. Docking was performed on the structure files of the compounds by using docking module PyRx. Binding energy was obtained as a result of the docking process that is shown in Table 1. These compounds were further analyzed for their Absorption, Distribution, Metabolism, Excretion and Toxicity properties. Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET):ADMET properties of chemical compounds were examined by online database named Danish Quantitative Structure-Activity Relationship (QSAR) database. Multicase Acute Aquatic Toxicity, Carcinogenicity, Arylhydroxylase Activity, Lethal Body Burden, Bioconcentration, Mutagenicity, Biodegradation, Environmental Partitioning and General Properties are included in ADMET properties. Forty compounds have affirmed ADMET properties using software but out of these forty compounds twelve compounds lastly fulfill maximum of the characteristics of drug target hopefuls. Thus, binding energy and ADMET of those twelve compounds have been shown in the outcome tables (Table:1 and Table:2).

Figure 1. *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

RESULTS AND DISCUSSION

In this work total ninety chemical compounds were screened as drug target candidate for *Dengue Virus* protein 4 NS3. Chemical and physical properties of these compounds were collected from chemical database. These properties such as pKa value, Molecular weight(MW), its state, Polarizibility, Melting and Boiling Point play essential role in the drug molecule(Dias et al., 2008). A weak acid incorporates a pKa worth within the scope between -2 to 12 and pKa values over twelve area unit are said to be alkaline drug. Acid with a pKa worth of less concerning -2 area units are said to be a strong acid. Similarity score denotes relevance of the new chemical with the chemical which may fit the drug target pocket. Polarizability shows the ability of a molecule to be polar; it is dependent upon the charge distribution and effects the bond formation in a chemical compound. Docking results denote that lower binding energy show the higher affinity of the drug target candidate. –5 to -9 Kcal/Mol is the normal range of binding energy(Modis, Ogata, Clements, & Harrison, 2003).

Every drug must comply with its ADMET properties. Amongst all their properties Mutagenicity, Toxicity, In-vitro test and Carcinogenicity are important properties of drug candidates to be examined. Preferably these properties are examined by tests like Ames test, Food and Drug Administration's Center for Drug Evaluation

and Research (FDA- CDER) properties on RAT and Mouse, Lethal Concentration, Lethal Dose in human, and Hypoxanthine-guannine phosphoribosyltransferase (HGPRT) test. These properties can also be predicted using ADMET software(Yang & Chen, 2004). Table 2 enlists skin irritation, skin sensitization, Respiratory sensitization and other properties of these molecules. Most of the molecules show negative skin sensitization, skin irritation, respiratory irritation and negative Ames and Hypoxanthine-guannine phosphoribosyltransferase (HGPRT) test indicates that these molecules can be considered as suitable hits for further drug design. Ames test can be utilized to check mutagenicity of the drug. Positive result indicates that the drug will be the mutagenic(Wadood et al., 2013; Williamson, 2014). CHOLICACID – (DB02659),MESTEROLONE – (DB13587)chemical compounds demonstrate positive Ames test, thereupon they are mutagenic and can only be used as drug target candidates after bringing changes in their molecular structure. Remaining compounds show negative Ames test; thus they are not mutagenic and can be considered as drug target candidates.

Certain tests such as-CDER Proprietary Male Rat, FDA Cancer Male Rat, FDA Cancer Female Rat, FDA Cancer Male Mouse, CDER Proprietary Male Mouse, FDA Cancer Female Mouse, -CDER Proprietary Female Rat, -CDER Proprietary Female Mouse are used to determine carcinogenicity. Positive result denotes that the drug will be the carcinogenic(Wadood et al., 2013; Williamson, 2014). TRICOSANIC ACID – (DB03500), chemical compounds indicate positive carcinogenicity thence they can only be considered as drug candidates after modification in the structure. Remaining compounds show negative carcinogenicity thus they are not carcinogenic and can be considered as drug target candidates. Lethal dose is a way to determine the short term poisoning potential of the material. Lethal concentration values solicit the concentration of the chemical. This toxicity is established on lethal concentration of the drug. The range of lethal concentration 100 – 1000mg/L indicates moderately toxicity of compound and based on that concentration lethal dose is set as 0.5-5 gm/kg.

If lethal concentration range is 10 -100 mg/L then it indicates highly toxicity of the substance and hence lethal dose has to be 5-50 mg /kg. If lethal concentration range is 1000 – 10000 mg/L then it indicates slightly toxicity of the compounds and lethal dose is set as 5 – 15 gm/ kg(Wadood et al., 2013; Williamson, 2014). Here screened each of the twelve compounds have lethal concentration range 100 – 1000 mg/L thus they are sensibly toxic and their lethal dose should be 0.5 – 5 gm/Kg. After evaluating physical and chemical properties, docking and ADMET analysis of all

Table 1. Showing Binding Energy of Chemical Compounds

Drug Accession Number	Binding Energy kca/mol
DB00825	-5.6
DB02202	-3.7
DB02659	-8.6
DB02854	-8.6
DB02901	-7.3
DB03017	-4.5
DB03193	-4.6
DB03500	-5.6
DB03619	-8.6
DB06777	-8.3
DB11789	-8.6
DB13587	-8.0

Table 2. Absorption, Distribution, Metabolism, Excretion and Toxicity Properties

	Health End Point			Mutagenicity	In Vitro Tests	Carcinogenicity
	Severe skin irritation	Skin sensitization	Respiratory sensitization	Ames test (Salmonella)	HGPRT	
METHANOL – (DB00825)	NEG	NEG	NEG	NEG	NEG	NEG
1,3BUTANEDIOL – (DB02202)	NEG	NEG	NEG	NEG	NEG	NEG
CHOLICACID – (DB02659)	NEG	NEG	NEG	POS	NEG	NEG
LAURIC ACID – (DB03017)	NEG	NEG	POS	NEG	NEG	NEG
STERIC ACID – (DB03193)	NEG	NEG	POS	NEG	NEG	NEG
AETIOCHOLANOLONE (DB02854)	NEG	NEG	POS	NEG	NEG	NEG
TRICOSANIC ACID – (DB03500)	NEG	NEG	NEG	POS	NEG	POS
DEOXYCHOLIC ACID – (DB03619)	NEG	NEG	NEG	NEG	NEG	NEG
CHENODEOXYCHOLIC ACID – (DB06777)	NEG	NEG	NEG	NEG	NEG	NEG
STANOLONE – (DB02901)	NEG	NEG	NEG	NEG	NEG	NEG

(* POS = POSITIVE) (* NEG =NEGATIVE)

Figure 2: Showing the Docking Pose of METHANOL (DB00825) with Dengue Virus 4 NS3 Helicase in complex with ssRNA

Figure 3: Showing the Docking Pose of 1,3BUTANEDIOL (DB02202) with Dengue Virus 4 NS3 Helicase in complex with ssRNA

Figure 4: Showing the Docking Pose of CHOLICACID (DB02659) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

Figure 5: Showing the Docking Pose of AETIOCHOLANOLONE (DB02854) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

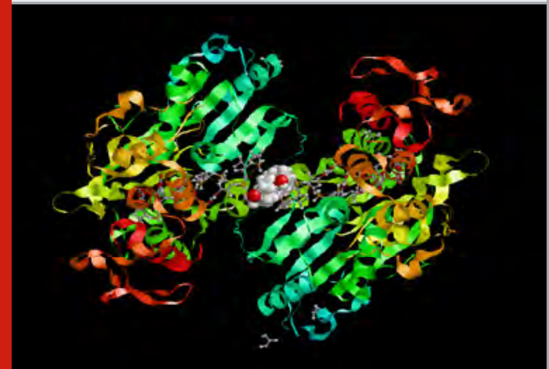


Figure 6. Showing the Docking Pose of STANOLONE (DB02901) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

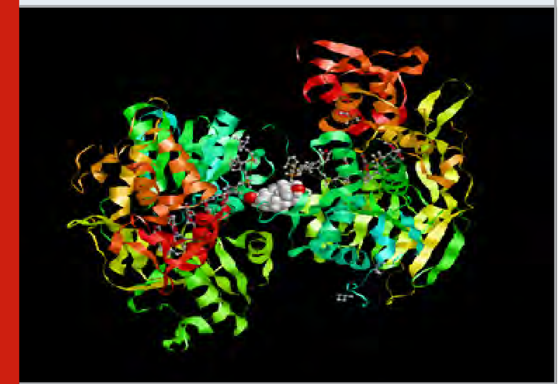


Figure 7. Showing the Docking Pose of LAURIC ACID (DB03017) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

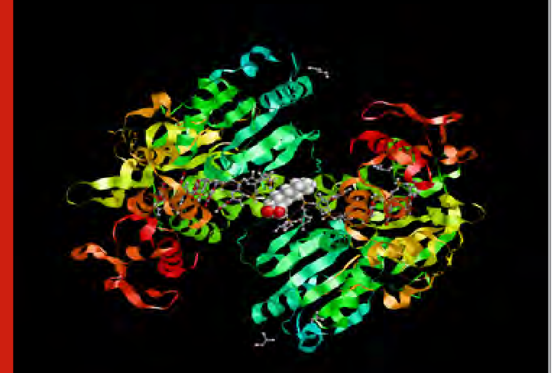


Figure 8. Showing the Docking Pose of STERIC ACID (DB03193) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

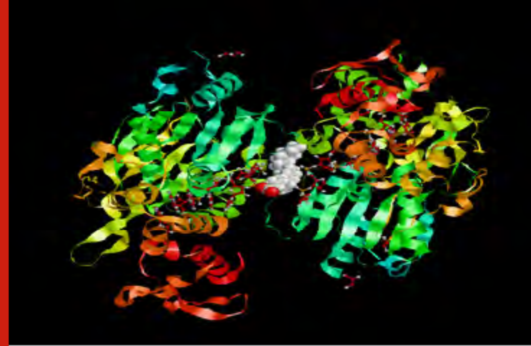


Figure 9. Showing the Docking Pose of TRICOSANIC ACID (DB03500) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

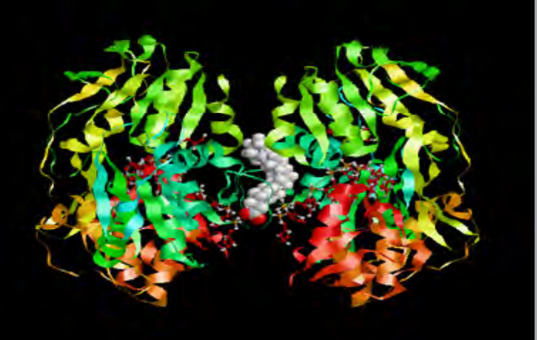


Figure 10. Showing the Docking Pose of DEOXYCHOLIC ACID (DB03619) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

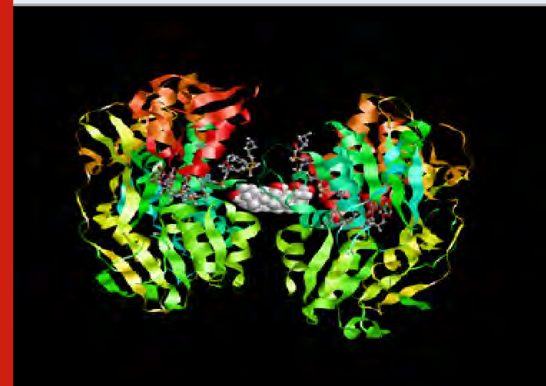


Figure 11. Showing the Docking Pose of CHENODEOXYCHOLIC ACID (DB06777) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

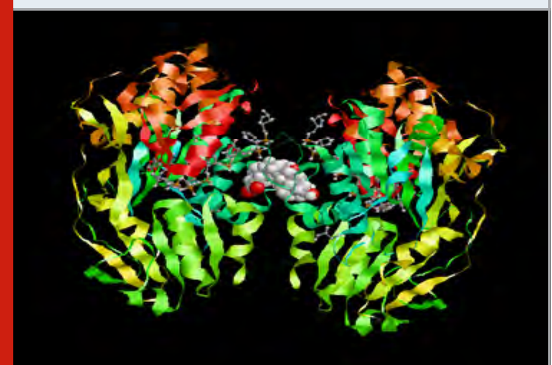


Figure 12. Showing the Docking Pose of HYDEOXYCOLIC ACID (DB11789) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA

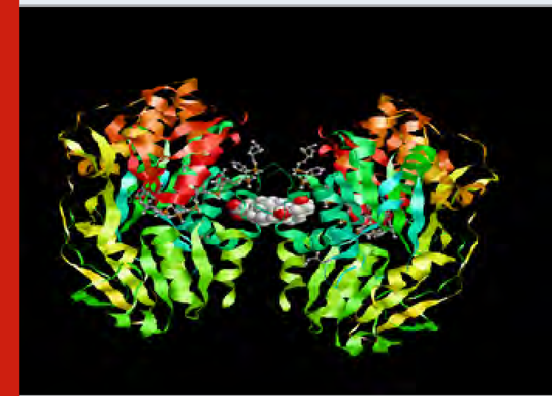
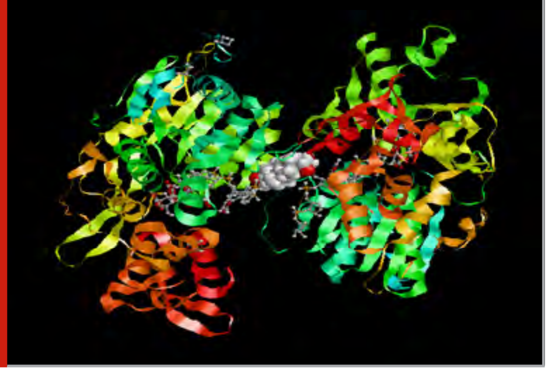


Figure 13. Showing the Docking Pose of MESTEROLONE (DB13587) with *Dengue Virus* 4 NS3 Helicase in complex with ssRNA



the screened chemical compounds we validate below chemical compounds as potential lead molecules for Dengue Virus4 NS3 protein. METHANOL – (DB00825), 1,3BUTANEDIOL – (DB02202), CHOLICACID – (DB02659), LAURIC ACID – (DB03017), STERIC ACID – (DB03193), AETIOCHOLANOLONE– (DB02854), TRICOSANIC ACID – (DB03500), DEOXYCHOLIC ACID – (DB03619), HENODEOXYCHOLIC ACID– (DB06777), STANOLONE – (DB02901), HYDEOXYCOLIC ACID – (DB11789), MESTEROLONE – (DB13587). Chemical Compounds apart from these are missing in above criteria. Thus, those chemical compounds can't be employed in present form as drug candidate for the same target.

SUMMARY AND CONCLUSION

Dengue Virus protein replicates in the host cells. NS3 Helicase in complex with ssRNA contains ligand named Glycerol, (propane-1,2,3-triol). It was identified as a drug target protein. Its structure was downloaded from the Protein Data Bank. Ligand Glycerol, (propane-1,2,3-triol) was obtained for further analysis. Similar structure search of different chemical compounds was done which were related to Glycerol, (propane-1,2,3-triol). At the end, 90 structures were obtained with their physical and chemical properties. Further, docking and screening were performed. ADMET study was done at the end of ADMET analysis and forty Chemical compounds were found to affect

protein target. Further, all parameters of those forty chemical compounds were analyzed and following twelve chemical compounds confirm their potential as drug target candidates.

METHANOL – (DB00825),1,3BUTANEDIOL – (DB02202), CHOLICACID – (DB02659), LAURIC ACID – (DB03017), STERIC ACID – (DB03193), AETIOCHOLANOLONE– (DB02854), TRICOSANIC ACID – (DB03500), DEOXYCHOLIC ACID – (DB03619), CHENODEOXYCHOLIC ACID– (DB06777), STANOLONE – (DB02901), HYDEOXYCOLIC ACID – (DB11789), MESTEROLONE – (DB13587). From the above twelve compounds these three are hormone derivatives: AETIOCHOLANOLONE– (DB02854), STANOLONE – (DB02901), MESTEROLONE – (DB13587). Thus, it can be concluded that above twelve chemical compounds show great affinity against *Dengue Virus* Protein NS3 Helicase in complex with ssRNA. Hence these twelve chemical compounds can be further analyzed as drug target candidates for dengue fever disease in wet lab for the Production of new synthesis of drug against dengue fever disease.

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Conflict of Interest Statement: We, the authors of the submitted manuscript declare that the work and data present in the manuscript entitled - Design and prediction of Absorption, Distribution, Metabolism, Excretion and Toxicity properties of drugs for dengue fever using in-silico approaches is genuine research carried out by us. The work finally belongs to the institutes. We have not misused the data previously published and have not manipulated the original work.

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The Relationship Between Serum Polyunsaturated Fatty Acid Ratio and Functional Status in Patients with Stroke Induced Sarcopenia

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ABSTRACT

Though an optimal serum polyunsaturated fatty acid ratio has yet to be defined, the identification of serum polyunsaturated fatty acid ratio as a clinical biomarker may have important implications for stroke recovery. The purpose of this study was to quantify habitual serum polyunsaturated fatty acid ratio to determine their relationship with physical function in a cohort of chronic adult stroke survivors. Twenty chronic stroke survivors (age: 61±9 years; BMI: 29±8 kg/m2; mean ± SD) were assessed for two-minute walk distance (2MWD). Plasma lipid and glucose profiles were measured, and Hs-IR calculated. Serum polyunsaturated fatty acid ratio profiles were assessed by mass spectrometry. Results: Average serum omega-6 concentrations were 164+41 µg/d and serum omega-3 (EPA+DHA) concentrations were 163±26 µg/d, with an omega-6 (AA) to omega-3 (EPA+DHA) serum concentration ratio of 1.6±0.3. These data suggest that the concentration ratio of serum polyunsaturated fatty acid may be important indices of physical dysfunction in patients with stroke induced sarcopenia.

KEY WORDS: STROKE, SERUM POLYUNSATURATED FATTY ACID RATIO, PHYSICAL FUNCTION, SARCOPENIA.

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INTRODUCTION

Stroke is one of the leading causes of disability worldwide. Post-stroke declines in functional status is common and contribute to stroke being a leading cause of long-term disability (Feigin, V.L. 2019) Although sedentary activity is often implicated in these declines, less attention has been given to the role of dietary factors. A chronic a debilitating disease like stroke affects all the systems of human body. The long terms effects on the musculoskeletal system includes structural, metabolic and function loss, which is directly associated with the progression of disease and disability. The structural changes found in the effected muscles may be related to decreased synaptic communication leading to lesser motor unit (Scherbakov et al, 2013). In the long run these strokes induced changes in the muscle structure and its metabolic activities, leads to sarcopenia (Cruz-Jentoft et al, 2010), essentially a disease of old age, associated with poor quality of life and increased mortality rate in elderly population (Lathuilière et al, 2019).

In recent years, stroke related degenerative muscle changes have been found to be a leading cause of sarcopenia in this population. Studies to date implicate higher serum polyunsaturated fatty acid ratios in association with reduced physical functioning and elevated pro-inflammatory states in older adults and neurological deterioration following acute stroke. Despite being at elevated risk status of serum polyunsaturated fatty acid ratio of chronic stroke survivors is not well documented. Though an optimal ratio has yet to be defined, the identification of serum polyunsaturated fatty acid ratio as a clinical biomarker may have important implications for stroke recovery (Noguchi H et al, 2009). Therefore, the purpose of this study was to examine the relationship of serum polyunsaturated fatty acid ratio with physical function in patients with Stroke induced sarcopenia.

MATERIALS AND METHODS

Chronic stroke patients having a score of more than 21 on lower limb Fugl-Meyer Scale (Kwong et al, 2019) and capable of independently walking at least 200 meters without any aid were included for this study. Subjects having additional motor deficits including any vestibular or orthopedic disorders or bilateral cerebral lesions or aged above 70 years or having high risk cardiovascular disorder were excluded from the study. Based on the inclusion criteria twenty (20) subjects, who have been diagnosed with ischemic stroke of more than six-month duration, aged between 50–70 years were recruited from the Riyadh region. Informed consent was obtained from all the subjects before participating in this study. Participants were screened and their age, gender, height, weight and duration of onset of stroke were recorded. Severity of neurological impairment was determined by Fugl-Meyer scale (Sulliven et al, 2011).

Rivermead Mobility Index (RMI) (Collen et al,1991), which is a valid tool to asses functional mobility in the Stroke population and two-minutes' walk test a sub-maximal exercise test used to assess aerobic capacity and endurance, were used to assess the physical functioning (Hiengkaew et al,2012). Plasma lipid, and high sensitivity-C-reactive protein (hs-CRP) concentrations were determined by colorimetric method. Lipids were extracted from serum samples using a modified Bligh & Dyer lipid extraction protocol (Bligh EG, Dyer WJ, 2015). Targeted lipidomic assays were conducted on a QTRAP 5500 LC-MS/MS system to identify selective omega-3 and omega-6 lipid species by precursor ion scanning for the m/z values corresponding to the respective molecular weights. Statistical Analyses: All the data was tabulated, and descriptive statics was prepared using IBM SPSS (Version 22, Chicago, IL) software was used. Pearson correlation coefficients were used to determine relationships between serum polyunsaturated fatty acid ratio and physical functioning.

RESULTS AND DISCUSSION

A total of 20 subjects, including 11male and 9 females with a mean age of 61.4+4.1 and with mean onset of stroke of 9.7+5.3 months, have participated in the study. Out of twenty, twelve participants were affected on left side and remaining eight suffered from right sided stroke Participants undergoing treatment for hypertension and dyslipidaemia occurred in 78% and 84%, respectively. Despite 38% being treated for diabetes, all participants had elevated fasting plasma glucose (≥100 mg/dL) and elevated hs-CRP (>3 mg/L) was observed in 18% of the participants. Serum Fatty Acid Profiles: Average serum omega-6 concentrations were 164+41 µg/d and serum omega-3 (EPA+DHA) concentrations were 163±26 µg/d, with an omega-6 (AA) to

omega-3 (EPA+DHA) serum concentration ratio of 1.6±0.3. Higher serum polyunsaturated fatty acid ratio were associated with lower two-minute walk test, higher HOMA-IR and increased serum hs-CRP concentration (P's<0.05). Additionally, a higher serum polyunsaturated fatty acid ratio was associated with higher fasting glucose and insulin concentrations, while higher serum polyunsaturated fatty acid ratio were associated with lower aerobic capacity and endurance (P's<0.05).

DISCUSSION

Many studies have documented the decline in quality of life associated with stroke related derangement in muscle metabolism and functions. Despite it, prevention of muscle loss is not considered as essential in most of the clinical and rehabilitation guidelines for managing stroke (Quinn et al, 2009). In this regards our study will pave way by establishing a relationship between the long chain poly unsaturated fatty acid ratio and it's the physical and metabolic functions in this population (Garbagnati et al 2009). Previous studies have suggested beneficial effects of the nutritional supplement provided immediately after stroke, on body composition (Ha et al, 2010). In contrast to this, our study shows that, greater serum polyunsaturated fatty acid ratio may adversely influence physical function and a decline in the distance walking ability. This may be due the fact that the study by Ha et al used caloric rich fatty acid supplements which may have a utilized the protective effects of essential amino acids in stabilizing the muscle metabolism. This study supports data from non-stroke populations suggesting that higher systemic concentrations of omega-3, compared to omega-6s, protect against accelerated age and disease-associated decline of physical performance (Reinders I. et al, 2015). Despite studies identifying an association between acute post-stroke plasma fatty acid profiles and stroke recurrence, (Galan et al, 2010) there is further need to design studies to establish the beneficial effects of these fatty acids in improving muscle health and function.

Although our data are limited in this regard, a higher omega-3 membrane concentration may explain the associations between higher serum polyunsaturated fatty acid ratio profiles and physical dysfunction in stroke by altering cell membrane fluidity, enhancing nitric oxide-mediated vasodilation, and attenuating platelet aggregation (Zanetti M. et al, 2015) Further, there is some suggestion that the neuroprotective mechanism of action of omega-3s may occur through their effects on inflammation and oxidative stress; omega-6s produce eicosanoid products, which are more potent mediators of thrombosis and inflammation than similar products derived from omega-3s (Gutierrez et al, 2013). Although the exact mechanisms are unclear, these findings suggest that serum omega-3s might be important indicators to help attenuate and monitor the progression of stroke disability (Reinders et al, 2015).

Table 1. Participant characteristics and physical and metabolic functioning data

N=20	Mean SD
Gender (male/female) n	12/8
Age, years	61.4+4.1
Stroke Onset, months	9.7+5.3
BMI (kg/m2)	29±5.3
Fugl-Meyer scores (Lower extremity)	28+5.3
Rivermead Mobility Index score	4.3+3.8
Six Minute Walk Distance (m)	112+28
Total Cholesterol (mg/dl)	139+47
Triglycerides (mg/dl)	67+23
LDL Cholesterol (mg/dl)	88+35
HDL Cholesterol (mg/dl)	51+22
hs-CRP (mg/L)	4.9+3.6
Eicosapentaenoic Acid (µg/dL)	61+18
Docosahexaenoic Acid (µg/dL)	102+33
Arachidonic Acid (µg/dL)	164+41

Table 2. Relationships of Omega-6/Omega-3 Dietary Serum Concentrations with Physical Function and metabolic risk factors

Pearson Coefficients	RMI	2MWD (m)	Glucose (mg/dL)	Insulin (µU/mL)	hs-CRP (mg/L)
Serum Polyunsaturated Fatty Acid Ratio	-0.32*	-0.35*	0.21	0.31	0.42**

*P<0.05; **P<0.01. Key: RMI: Rivermead Mobility Index, 2MWD: Two-Minute Walk Test

Limitation: The results from this cross-sectional study were obtained from a small group of subjects; therefore, the results must be interpreted with caution. In addition, this study did not examine the effect of dietary poly unsaturated fatty acids and thus failed to determine a potential mechanism for the effectiveness of physical and metabolic function.

CONCLUSIONS

These data suggest that the concentration ratio of serum polyunsaturated fatty acid may be important indices of physical dysfunction in patients with stroke induced sarcopenia.

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